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Molecular Cytogenetics
- its use in human gene mapping and the detection
and definition of subtle chromosomal aberrations

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Thesis submitted to the Faculty of Medicine, University of Glasgow, for the
Degree of Doctor of Philosophy (Ph.D)

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DECLARATION

I certify that the work presented in this thesis has been performed by me, and that the results of this study have not been submitted for any other degree.

Norma Morrison

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Published articles

Reid E, Morrison N, Barron L, Boyd E, Cooke A, Fielding D, Tolmie JL (1996) Familial Wolf-Hirschhorn syndrome resulting from a cryptic translocation: a clinical and molecular study. *J Med Genet* 33:197-202

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Tobias ES, Morrison N, Whiteford ML, Tolmie JL (1999) Towards earlier diagnosis of 22q11 deletions. *Arch Dis Child* 81:513-514

Bryce LA, Morrison N, Hoare SF, Muir S, Keith WN (2000) Mapping of the gene for the human telomerase reverse transcriptase, hTERT, to chromosome 5p15.33 by fluorescence in situ hybridisation. *Neoplasia* 2:197-201

Posters

Morrison N, Tolmie J, Dunlop A, Davidson N, Jamieson A, Brewer C, Boyd, E. Chromosome 22q11.2 microdeletion: what are the differential diagnoses? Presented at the 1st European Cytogenetics Conference, Athens, June 1997.

Johnson DS, Morrison N, Ray K, Tolmie JL, Ryan E. Mild clinical phenotype associated with direct duplication of proximal 8p (46,XX,dup(8)(p11.1p21.1~p21.2)). Presented at the British Society for Human Genetics Conference, York, September 2004. *J Med Genet* 41(Suppl 1):S61, 2.40

Spoken presentations

Detection of a cryptic translocation (10p;18q) in a Newfoundland family with previously unexplained mental retardation. MacGregor D, Moore S, Morrison N, Connolly-Wilson M, Ives EJ. Presented by D MacGregor at the Canadian Association of Genetic Counsellors Annual Meeting, Halifax, Nova Scotia, Canada, December 2001.

Dean JCS, Morrison N, Tolmie J. Telomere deletions and learning disability - beginnings rather than endings. Presented by Dr JCS Dean at the Manchester Birth Defects Meeting, Manchester, November 2002.

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LIST OF ABBREVIATIONS

A	adenine
BAC	bacterial artificial chromosome
BMD	Becker muscular dystrophy
bp	base pair
BrdU	bromodeoxyuridine
BSA	bovine serum albumin
C	cytosine
CaCl ₂	Calcium chloride
CAF	conotruncal anomaly face
CCD	charge coupled device
CENPs	centromere proteins
CGH	comparative genomic hybridisation
CI	confidence interval
CK	creatine kinase
cM	centimorgan
cMpter	distance from the terminal short arm of a chromosome, in centimorgans
DAPI	4',6-diamidino-2-phenylindole
DGS	DiGeorge syndrome
DIG	digoxigenin
DMD	Duchenne muscular dystrophy
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
DOP	degenerate oligonucleotide primer
EDTA	Ethylenediamine tetraacetic acid
e.g.	exemplia gratia (for example)
EST	expressed sequence tag
et al	et alia
FISH	fluorescence <i>in situ</i> hybridisation
FITC	fluorescein isothiocyanate
FLpter	fractional length of the chromosome relative to the short arm terminus
G	guanine
HR-CGH	high resolution comparative genomic hybridisation
ISH	<i>in situ</i> hybridisation
IRS	interspersed repetitive sequences
ISCN	international system for human cytogenetic nomenclature
kb	kilobase pair
kDa	kilodalton
LCR	low copy repeat
LINES	long interspersed nucleotide sequences or long interspersed elements

MAPH	multiplex amplifiable probe hybridisation
Mb	megabase pair
M-FISH	multiplex FISH
MgCl ₂	Magnesium chloride
MIM	Mendelian inheritance in man
MLPA	multiplex ligation-dependent probe amplification
min	minute
MR	mental retardation
NA	numerical aperture
No.	number
NORs	nucleolar organising regions
OMIM	online Mendelian inheritance in man
PACs	bacteriophage P1-derived artificial chromosomes
PCR	polymerase chain reaction
PFGE	pulsed field gel electrophoresis
PGD	pre-implantation genetic diagnosis
PHA	phytohaemagglutinin
PND	prenatal diagnosis
PRINS	primed <i>in situ</i> labelling
RNA	ribonucleic acid
SD	standard deviation
SINES	short interspersed nucleotide sequences or short interspersed elements
SKY	spectral karyotyping
SRD	shortest region of deletion
SRO	shortest region of overlap
SSC	standard saline citrate
STS	sequence tagged site
T	thymine
Taq	<i>Thermus aquaticus</i>
Taq IS	AmpliTaq IS (<i>in situ</i>)
T _m	melting temperature of double-stranded DNA
TOF	tetralogy of Fallot
TRITC	Tetramethyl rhodamine isothiocyanate
TSA	tyramide signal amplification
U	unit
UV	ultraviolet
VCFS	velocardiofacial syndrome
VSD	ventricular septal defect
WS	Williams syndrome
YAC	yeast artificial chromosome

SUMMARY

This work involved the investigation of three different molecular cytogenetic approaches, Fluorescence *In Situ* Hybridisation (FISH), Comparative Genomic Hybridisation (CGH) and Primed *In Situ* (PRINS) labelling, and their utilisation in chromosomal localisation of DNA sequences, breakpoint definition and the detection of cryptic abnormalities.

As a development of earlier mapping in this department using non-fluorescent ISH, simple and reliable localisation, by FISH, of unique cloned sequences in a range of vectors was optimised and applied. Among the sequences localised were myotonic dystrophy protein kinase-related Cdc42-binding kinase β (*MRCK β*) and telomerase reverse transcriptase (*hTERT*), to 14q32.31~q32.32 and 5p15.33 respectively.

Probes were sourced, and their map localisations confirmed or refined, for application to two cases of chromosomal abnormality which required breakpoint definition. The first was a patient with der(12)(12qter→12p13.3::21q22.3→21q11.2::21q11.2→21qter).

Breakpoint mapping with 21q-specific clones showed that the 21q region monosomic in this individual was proximal to BAC sequence 268F23, which lies at 16.03Mb from 21pter, and included marker D21S1911 at 15.06Mb from 21pter. The breakpoint of at least one of the two chromosome 21 components of the derivative chromosome 12 lies between these two loci. This has provided a focussed region for further biochemical and molecular study.

The second case was a rare proximal duplication of 8p with a mild phenotype. The duplication was confirmed to be direct, rather than inverted, and the breakpoints defined as dup(8)(p11.1p21.1~p21.2). This abnormality had not previously been reported.

Concurrently, FISH probes were sourced, extracted and optimised for use in diagnostic investigation of cryptic segmental aneuploidy in DiGeorge/velocardiofacial (DG/VCF), Rubinstein-Taybi and Wolf-Hirschhorn syndrome patients, and in potential carriers of Duchenne and Becker muscular dystrophy.

Chromosome 22q11.2-specific cosmids were used to investigate 161 patients with features of DiGeorge/velocardiofacial syndrome, detecting 34 deletions. Following reports of hemizygosity at 22q11.2 in patients with non-syndromic conotruncal defects, a series of 24 patients with tetralogy of Fallot were examined for microdeletion at this locus and 4 deletions were disclosed.

Plasmid probes for 4p16.3 and 11p15.5 were used to determine a cryptic t(4;11) rearrangement in a child for whom there was clinical suspicion of Wolf-Hirschhorn syndrome. This provided a means to carrier determination and prenatal diagnosis for her large, extended family.

Cosmids specific for 16p13 were used for FISH assessment of a group of 15 patients with suspected Rubinstein-Taybi syndrome. No microdeletions were found with the original RT1 cosmid probe, but work now continues with 4 additional cosmids which span the Rubinstein-Taybi syndrome critical region.

Prior to and during the introduction of a quantitative PCR method in this department a range of 20 exon-specific dystrophin probes was used to provide an unequivocal, simple method of carrier determination in families with inherited microdeletion at Xp21. Ninety seven female carriers were diagnosed in a total of 83 families using this FISH approach. Following the report that ~6% of cases of idiopathic mental retardation might be associated with a cryptic subtelomeric rearrangement (Flint et al 1995), the novel commercial Multiprobe-T device was initially appraised then used to screen a series of 100 patients. This disclosed three cryptic chromosomal abnormalities, namely a der(9)t(3;9), der(9)t(9;16) and der(18)t(10;18).

Successful clinical application by Bryndorf et al (1995) prompted optimisation and assessment of CGH in the investigation of a selection of patients with apparent or suspected constitutional chromosomal aberrations. Abnormalities detected included a subtle deletion at 17p11.2, and a deletion involving 1q25-q31 in an individual with an apparently balanced translocation of chromosomes 5 and 6 but abnormal phenotype. In combination with FISH, CGH refined characterisation of an apparent but unresolvable abnormality of chromosome 3, demonstrating duplication of 3p24.2-p25 and deletion of the 3p subtelomeric region.

This study also involved the introduction of PRINS technology to this department and evaluation of its potential for robust detection of unique target sequences. Technical modifications assessed included, proteinase K and ligase pretreatments and use of Tyramide Signal Amplification (TSA). Slide pretreatment in Carnoy's fixative then 2xSSC, and use of an alternative, relatively inexpensive, enzyme not previously reported in PRINS (Dynazyme), were identified as beneficial.

PRINS with commercial repetitive PRINS kits and 'in house' repetitive sequence primers was successful, but no satisfactory results were obtained with primers for unique or very low copy sequences.

The future role of these methods in mapping and investigation of segmental aneusomy is discussed and array CGH, a high resolution refinement of CGH, reviewed.

1. INTRODUCTION

1.1 General Introduction

Historically genetic diseases have been diagnosed by biochemical analysis of gene products, cytogenetic study of visible chromosomal changes and, most recently, molecular study of DNA itself. Within cytogenetics one aim has been to improve resolution of subtle aberrations and to define breakpoints. Molecular cytogenetics, combining cytogenetic and molecular techniques, has, though initially employed in sequence localisation, come to provide major advances in the scope and utility of diagnostic chromosome investigation.

1.2 DNA and the Chromosome

Genetic information is carried in DNA, as triplets of nitrogenous bases adenine (A), guanine (G), thymine (T) and cytosine (C) in two nucleotide chains. These form a double helix joined by hydrogen bonds between the inwardly projecting bases.

DNA is packaged in eukaryotic chromosomes as chromatin, a mix of DNA, protein and associated RNA organised on four basic levels. The elementary DNA fibre, 10nm in diameter, consists of repeating nucleosome units constructed from histone molecules (two each of H2A, H2B, H3 and H4) around which 200 base pairs (bp) of DNA is coiled 1.75 times. This is coiled into a chromatin fibre, 30nm in diameter, and loops of fibre (Laemli loops), each about 200 kilobase pairs (kb), radiate to form the 0.6µm diameter body of the chromatid. Further chromatid coiling produces the quaternary structure of the chromosome.

Humans have 24 types of nuclear chromosomes: 22 autosomes (as two copies in somatic cells but one in gametes) an X chromosome (two in female but one in male somatic cells) and a Y (in males). In late prophase or metaphase stages of cell division, mitosis, cells spread on glass slides and visualised by light microscopy have condensed chromosomes with differential morphologies. The 'karyotype' is the collective features of a cell's chromosomes or an image of these stained, paired and ordered numerically.

Chromosomes are constructed of two broad classes of DNA - heterochromatin and euchromatin, and within the chromosome there are recognisable substructures including centromeres and telomeres.

Heterochromatin and Euchromatin

Heterochromatin is highly contracted, tends to be transcriptionally inactive, and replicates late. Facultative heterochromatin is formed on condensation and inactivation of euchromatin (as with one X chromosome in female mammalian cells). Constitutive heterochromatin generally flanks centromeres and telomeres, though its amount and distribution varies between individuals, and is enriched with repetitive sequences. Euchromatin contains the unique or low-copy sequence structural genes and related regulatory sequences, and is assumed to be genetically active and less contracted than heterochromatin.

The proportions of repetitive and unique sequence DNA in a genome can be determined from a 'cot' curve (Fig.1-1).

Centromeres

The centromere is generally the site of the primary chromosomal constriction, the location of the kinetochore (the anchor point for microtubule proteins of the mitotic spindle) and the last point of sister chromatid pairing at anaphase, the penultimate stage in mitosis. It is composed of satellite DNA, predominantly alphoid, and associated proteins (CENPs). Alphoid repeats, though not necessarily the only essential component, appear pivotal to centromere function.

Telomeres

Telomeres are the structures which protect chromosome ends from exonucleolytic degradation, end to end fusion and irregular recombination. The highly conserved telomeric repeat sequence (TTAGGG)_n, represents 4-6kb in the somatic cells of human neonates. Telomere repeats minimise the effect of the 'end replication problem' (Watson 1972) which results in shortening of the chromosome at each cell division. This is caused by the inability of the DNA replication system to copy the 5' terminus of DNA, resulting in one strand being fully synthesised while the other has an unsynthesised section at the end on which the DNA polymerase is primed. The terminal positioning of non-coding repeats means it is these, rather than the rest of the chromosome, which shorten, by an average of 31bp per year (Slagboom et al 1994).

Telomeres are maintained by the riboprotein telomerase. This acts as a reverse transcriptase, using the 3' end of the G-rich strand as a primer to synthesise telomeric DNA repeat units from its integral RNA template (Morin 1989). Human telomerase activity is high in most tumour, germ-line and stem cells (which are capable of infinite or prolonged proliferation) but low in somatic tissues, suggesting that telomere instability may contribute to senescence (Counter et al 1992).

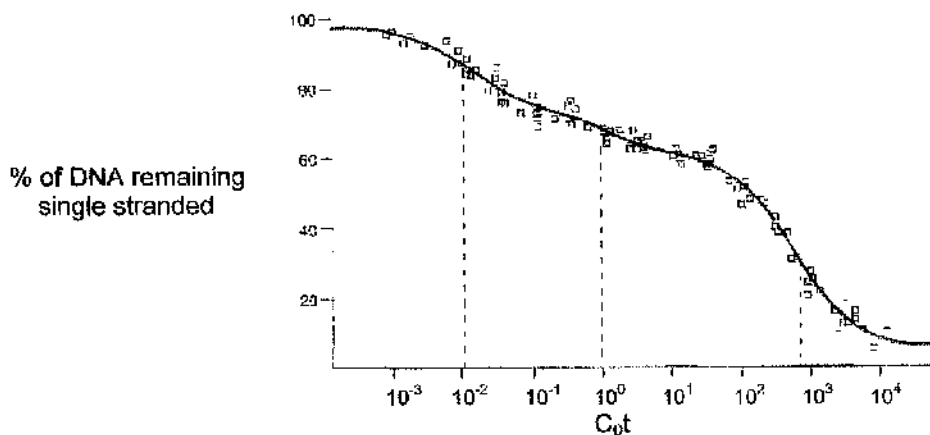


Fig.1-1 A 'cot' curve displaying the renaturation kinetics of sheared, denatured human DNA. Dashed lines indicate approximate $C_0t_{1/2}$ values for highly repetitive, moderately repetitive and single copy DNA classes. Adapted from McConkey (1993).

The proportion of repetitive DNA in a genome can be assessed by tracking the rate at which denatured DNA becomes double-stranded under standard conditions: the more copies of a given sequence, the faster they reassociate. The fraction of DNA still single-stranded is measured after different incubation periods. Results are then graphed as a plot of C/C_0 against the logarithm of C_0t where C is the concentration of single stranded DNA remaining after t seconds of incubation and C_0 its concentration at time zero. The human DNA 'cot' curve comprises the sum of the individual cot curves of various kinetic classes of DNA that renature at different rates, and the estimated plateaus of the individual curves gives the percentage of each class in the total DNA.

1.3 Cell and Chromosome Division

1.3.1 Meiosis and Mitosis

Chromosome division takes two forms. In meiosis, which occurs during gametogenesis, there are two divisions (I and II) with only one replication (prior to meiosis I), so the haploid number of chromosomes (23) passes to each of four daughter cells.

Recombination between maternally and paternally derived chromosomes and independent assortment of chromosomes into gametes takes place. In mitosis the genetic material of a cell replicates then is divided equally between two daughter cells.

1.3.2 The Mitotic Cycle

The mitotic cycle in humans takes 24 hours. For most of the cycle the cell is in interphase, the G_1 (G_1) interphase stage, immediately after telophase, lasting longest. Each chromosome exists at that time as a single thread of double-stranded DNA, or chromatid. Chromosome replication subsequently occurs during the S phase (which lasts 6-8 hours). By G_2 chromosomes have two chromatids.

The remaining mitotic stages, i.e. prophase and metaphase (when chromatin coiling produces visible chromosomes), then anaphase and telophase, take 20 to 60 minutes.

1.4 Human Cytogenetics

1.4.1 A Brief History

Walter Flemming, who introduced the term 'mitosis' in 1882, was reputedly the first to observe chromosomes. Wilhelm Waldeyer-Hartz named them 'chromosomes' (coloured bodies) in 1888.

Modern human cytogenetics began in 1956, when tissue culture and squash techniques, colchicine mitotic arrest and chromosome spreading with hypotonic salt solutions were simultaneously available and allowed determination of the human chromosome number as 46 (Tjio and Levan 1956). Drying of fixed chromosomes on microscope slides provided further improvement in metaphase quality (Rothfels and Siminovitch 1958).

However, diagnostic cytogenetics only became relatively routine when the use of phytohaemagglutinin (PHA) to stimulate T lymphocyte mitosis allowed chromosomes to be obtained from cultures of easily accessible peripheral blood (Moorhead et al 1960).

The first chromosomal disease to be demonstrated was trisomy of a small chromosome, later called 21, in Down Syndrome (Lejeune et al 1959). Absence of an X chromosome, and no Y, was soon reported in females with Turner syndrome (Ford et al 1959) and the first successful prenatal diagnosis (PND), for trisomy 21, was performed in 1965 (Steele and Breg 1966).

Although such numerical, and also gross structural, chromosome disorders could be recognised, precise pairing and identification of all chromosomes only became possible on the introduction of staining techniques which produced a series of light and dark bands. Fluorescent staining with quinacrine was the first (Caspersson et al 1970) followed by many alternative methods, including G-banding (Drets and Shaw 1971, Seabright 1971). Synchronised culture techniques were used to obtain prometaphase chromosomes and their banding made it possible to determine chromosome segments and breakpoints even more accurately by greatly increasing, to 1000-2000, the number of visible bands (Yunis 1976).

1.4.2 Chromosome Banding Techniques

Chromosome banding techniques can be considered in two categories, those designed for differential identification of all chromosomes and those which highlight specific chromosome features (references and full methods in Verma and Babu 1995).

Techniques used in human cytogenetics for differential identification of all chromosomes include fluorescent, Giemsa and reverse banding.

Fluorescent banding

Here fluorochromes intercalate DNA and produce banding visualised by fluorescence microscopy with appropriate optical filters. Q-banding involves quinacrine dihydrochloride. Alternative stains such as DAPI (4,6-diamidino-2-phenylindole), Hoeschst 33258 and acridine orange have been used alone or in combination to produce different banding profiles, but fluorescent banding is disadvantaged by relatively low resolution and fluorophore fading.

Giemsa (G- or GTG-) banding

This produces permanent bands which are highly suitable for diagnostic work.

Chromosome pretreatment is with salt solution at 60°C or protease, usually trypsin (Drets and Shaw 1971, Seabright 1971) and staining is with Giemsa, Leishman or Wrights stain. Brightly fluorescent Q-bands appear dark and dull Q-bands light with this method. G-light bands replicate earlier than G-dark bands, which are 3.2% richer in A and T sequences. Depending on chromosome length, 300-2000 bands can be produced (per haploid set) with this method though the band number is usually 400-800 in routine work.

Chromosomes and their G-bands are described and numbered in an international nomenclature system (ISCN 1995). Chromosomes are, mainly, numbered by size (the largest being 1) and categorised as metacentric, acrocentric or submetacentric according to centromere position (Fig.1-2). The shorter and longer arms of a chromosome are called petit (p) and queue (q). Bands on each arm are numbered distally from the centromere.

Reverse (R-) banding

Pretreatment with alkali at 80-90°C and staining with Giemsa (RHG bands) or fluor such as acridine orange (RFA bands) produces banding that is the reverse (dark bands become light and vice versa) of G- or Q-banding respectively. This method, and a modification, T-banding, can stain chromosome ends darkly, assisting their resolution.

Techniques which identify specific chromosome features include 5-bromodeoxyuridine (BrdU) staining, which is used in order-of-replication and sister chromatid exchange studies, centric (C-) banding which highlights heterochromatic regions around the centromeres and on chromosome Y, and staining, involving acid treatment, of the nucleolar organising regions (NORs) on the short arms of acrocentric chromosomes 13, 14, 15, 21, and 22 (Fig.1-2). Active NORs are stained using silver nitrate.

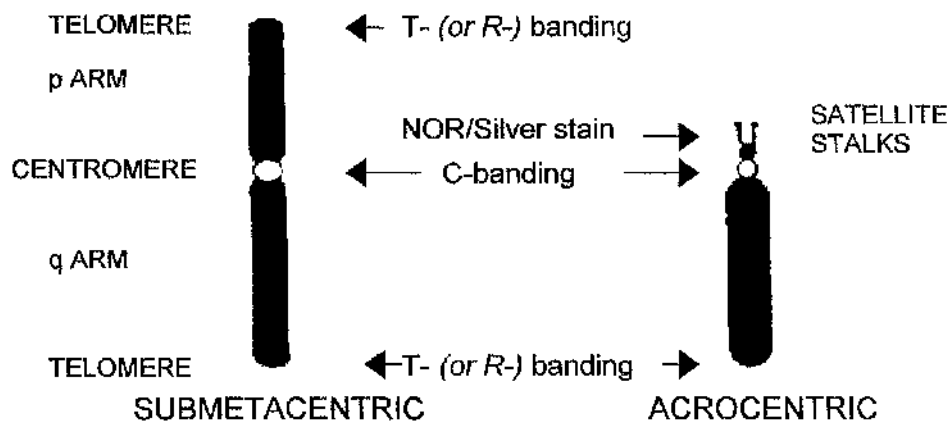


Fig.1-2 Position of C-, T- (or R-) bands and NOR stain on submetacentric and acrocentric chromosomes. In submetacentric chromosomes the p arm is shorter than the q arm.

1.4.3 Types of Chromosomal Aberrations

Chromosome aberrations affect at least 7.5% of conceptions and 0.6% of live births. These can be numerical (varying from the diploid number) or structural and occur either as a result of a germ cell mutation in an ancestor or due to somatic mutation, in which case only a proportion of cells will be affected (mosaicism). Detailed discussion of human chromosome abnormalities is provided in Gardner and Sutherland (1996).

Numerical Aberrations

Aneuploidy is any chromosome number which is not an exact multiple of the haploid number. It usually arises from the failure of paired chromosomes or sister chromatids to disjoin at anaphase (non-disjunction) or delayed chromosomal movement at anaphase (anaphase lag) and can occur during mitosis or meiosis I or II. Non-disjunction during mitosis may cause mosaicism - the presence, in an individual derived from a single zygote, of two or more cell lines with differing chromosomal complements. The only autosomal aneuploidies occurring at appreciable frequencies in live births are trisomy 13, trisomy 18 and most commonly trisomy 21 (1 in ~700 births). The only known autosomal

monosomy is the rare monosomy 21. Sex chromosome aneuploidies with appreciable incidence include 45,X0, 47,XXX, 47,XXY and 47,XYY.

Polyploidy is an abnormal number of complete haploid sets (i.e. more than two). Triploidy (three times the haploid number, n) may arise by various processes including fertilisation of the egg by two sperm. Triploids represent around 17% of spontaneous abortions and rare cases which survive the perinatal period are usually mosaic, having a diploid cell line. Tetraploidy ($4n$), is found in around 6% of spontaneous abortions.

Structural Aberrations

The variety of structural aberrations, caused by random re-joining of broken chromosome ends, is limited only by the possible lethal effect of any rearrangement. Rates of breakage vary according to genetic make-up and age, and are increased by exposure to various radiations, mutagenic chemicals, viruses, and in individuals with chromosome instability syndromes (e.g. Fanconi anaemia). Partial trisomy and/or monosomy can be caused by translocations, deletions, duplications, ring chromosomes, inversions, isochromosomes and centric fragments (Table 1-1, Fig.1-3). Of particular relevance to this study are reciprocal translocations, deletions and duplications.

Translocations

Here transfer of material between chromosomes is caused by breakage of more than one chromosome followed by incorrect repair, or by erroneous meiotic recombination between non-homologous chromosomes. Where no genetic material is lost, and no critical locus disturbed, the affected individual may be clinically normal but have a risk of chromosomal imbalance in any progeny.

Simple reciprocal translocations arise when a two-way exchange of material occurs between two non-homologous chromosomes. The rearranged, or derivative, chromosomes are named according to the centromere carried. Around 1 in 625 individuals is a carrier of a familial or *de novo* reciprocal translocation. The risk to a carrier of abnormal offspring and/or miscarriage depends on the chromosome segments translocated (Jalbert et al 1980).

Deletions

Deletions arise from loss of a chromosome segment distal to a single break (terminal), between two breakpoints (interstitial), as a consequence of unequal cross-over, or by inheritance of an unbalanced form of a parental translocation. The smallest cytogenetically visible deletion, 4Mb (Connor and Ferguson-Smith 1993), represents loss

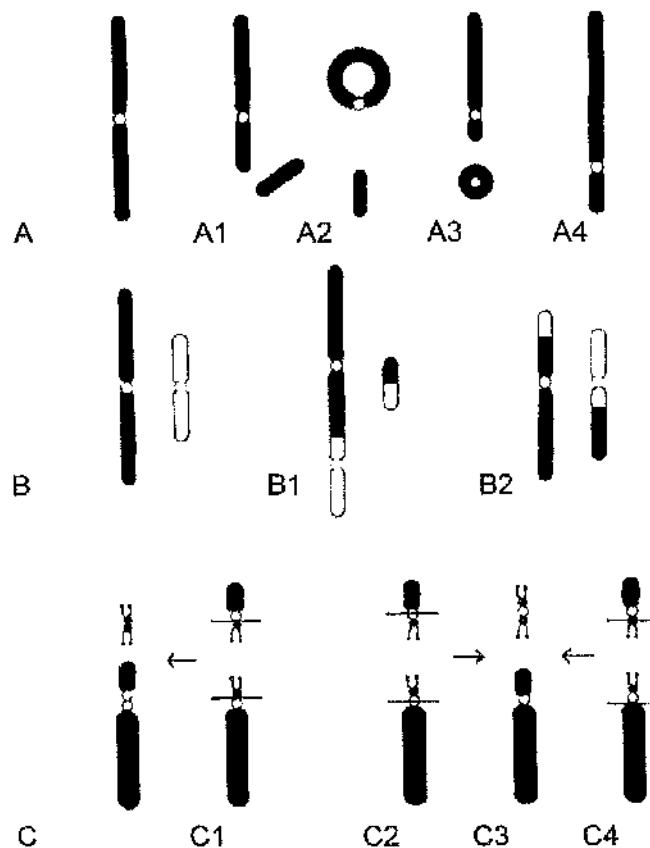


Fig.1-3 Chromosome breaks and rearrangements.

Adapted from Therman and Susman (1993).

Result of G_1 breaks in one chromosome (A), two chromosomes (B), and, forming Robertsonian translocations, breaks in two acrocentric chromosomes (C):

(A1) broken chromosome; (A2) centric ring and acentric fragment; (A3) acentric ring and centric fragment; (A4) pericentric inversion

(B1) dicentric chromosome and acentric fragment; (B2) balanced reciprocal translocation

(C1) breaks on the short arms forming a dicentric and acentric chromosome; break through the centromeres (C2) or one break on a short arm, one on long arm (C4) forming two monocentric chromosomes.

of large numbers of genes, therefore autosomal deletion usually results in multiple congenital abnormalities and mental retardation (MR).

Duplications

The presence of two copies of a chromosomal segment might arise through unequal cross-over in meiosis (the reciprocal product being deleted) or meiotic events in a parent with a translocation, inversion or isochromosome.

Duplications are generally less harmful than deletions (Connor and Ferguson-Smith 1993).

CHROMOSOME ABERRATION	CAUSE
Reciprocal Translocation	Two-way exchange of material between two non-homologous chromosomes
Robertsonian Translocation (centric fusion)	Breaks at or near the centromere in acrocentric chromosomes with translocation of the products usually forming a dicentric (with no NOR) and a non-viable acentric chromosome. Chromosome 21 involvement can cause familial translocation Down Syndrome.
Insertional Translocation	Three breaks in one or two chromosomes. If two involved, an interstitial deletion results in one with the deleted segment inserted in the gap of the other.
Deletion	Loss of a chromosome segment distal to a single breakpoint (terminal) or between two breaks (interstitial). See 'duplication'.
Ring Chromosome	Breaks in both arms of a chromosome and fusion of the resultant chromosome ends.
Duplication	Unequal cross-over in meiosis (reciprocal product being deleted) or meiotic events in a parent with a translocation, inversion or isochromosome.
Inversion	Two breaks in a single chromosome and, before re-joining, the intervening segment is reversed. Peri- or paracentric depending on whether the inverted segment does or does not involve the centromere. Generally of no phenotypic significance but the risk of unbalanced gametes increased.
Isochromosome	Deletion of one arm with duplication of the other, resulting either from transverse centromeric fission during cell division or from an isochromatid break and fusion above the centromere (with inactivation of one centromere in the resultant dicentric).
Centric Fragment	Centric fusion translocation between satellited chromosomes during meiosis. May have no clinical significance unless transcribed genes involved.

Table 1-1 Structural chromosome aberrations and their origins.

1.4.4 Limitation of Chromosome Banding Techniques

Many clinically significant chromosomal aberrations, such as microdeletions, are beyond the resolution of conventional banding, and its monochromatic nature can render interpretation, especially of complex rearrangements or small centric fragments, uncertain or impossible. In addition, identification of chromosome anomalies by banding techniques is not always appropriate or feasible. Crucially, the abnormality may not be present in dividing cells or high quality metaphase spreads may not be available (a particular problem in cancer cytogenetics). Another practical consideration is the time and skill level required for interpretation of high resolution banding.

These limitations prompted, during the 1990s, the evolution of *in situ* hybridisation techniques as an adjunct to classical diagnostic cytogenetics.

1.5 Development of Fluorescence *In Situ* Hybridisation

In situ hybridisation (ISH) is a method for the detection of specific nucleic acid sequences in specimens fixed to microscope slides, allowing determination of the presence and localisation of these sequences within a morphological context. The technique, when applied to chromosomes, represents a convergence of molecular genetics and cytogenetics thus the term molecular cytogenetics was adopted for the scientific field.

1.5.1 Isotopic ISH

Hybridisation of a nucleic acid sequence to the DNA of a cytological preparation was first described in 1969 (Gall and Pardue, John et al), when tritium-labelled ribosomal RNA was localised in *Xenopus* oocytes. Following acid fixation of the preparation to remove basic proteins which can interfere with ISH, the technique involved denaturing chromosomal DNA while retaining cytological integrity, then incubation with a single stranded probe sequence under conditions enabling specific pairing. A significant excess of silver grains in an autoradiographic emulsion overlay localised the site of hybridisation. The principles of this approach remain the basis of molecular cytogenetics today (Fig.1-4).

From the early 1970s this procedure was increasingly used to localise repetitive sequences, since RNA or DNA probes for these could be readily isolated (Saunders et al 1972, Jones et al 1973, Evans et al 1974). The parallel development of DNA recombinant technology made pure, single copy, sequences available and improvements in the efficiency of labelling, hybridisation and quality of chromosome banding eventually made ISH sufficiently sensitive to permit the localisation of such single copy sequence DNA (Gerhard et al 1981, Harper and Saunders 1981, Malcolm et al 1981). High specific activity tritiated probes could detect as little as 0.5kb of target sequence on pachytene chromosomes (Jhanwar et al 1983).

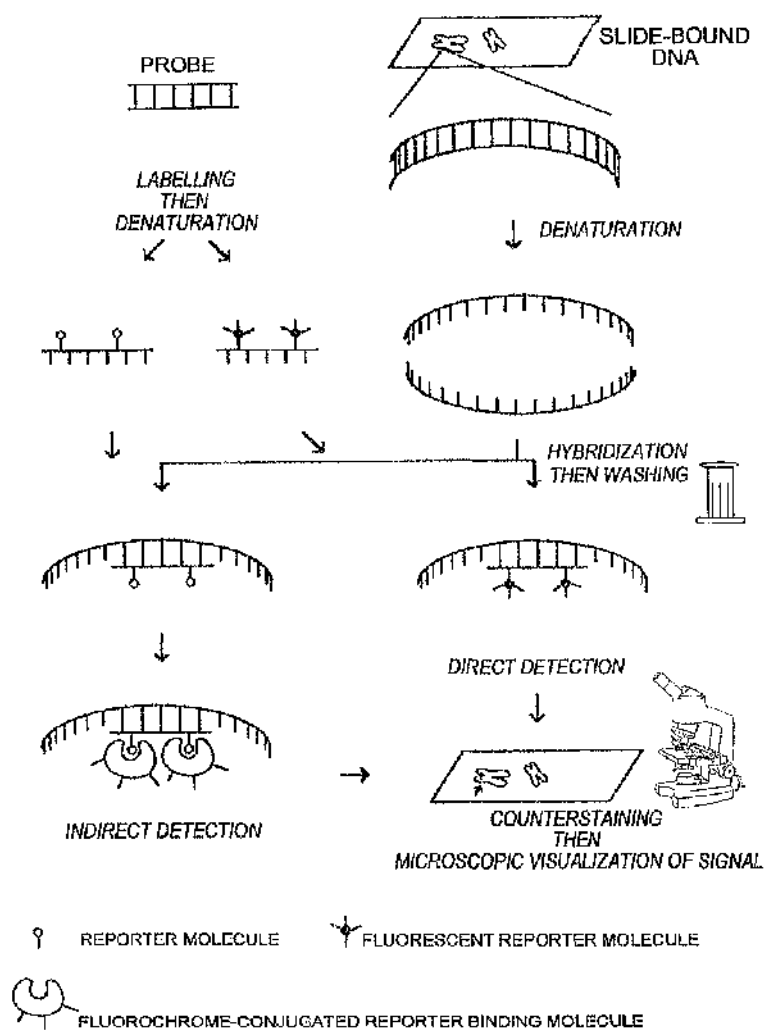


Fig.1-4 Stages of the FISH process. Adapted from Lichter and Cremer (1992).

1.5.2 Non-isotopic ISH

1.5.2.1 Labels and Labelling Methods

Isotopic ISH had, however, serious disadvantages. These included prolonged autoradiographic exposure of up to several weeks, poor resolution due to scattered radioactive disintegrations around the hybridisation region, probe instability caused by radioactive decay, the necessity for statistical analysis of a large number of metaphases to establish statistically significant counts above background level, and the risk to personnel involved in the use of radioisotopes.

The quest for faster, safer, more specific ISH effected, in the late 1970s to mid 1980s, development of several methods using non-isotopic probe labels, also known as reporter

molecules. Probe incorporation of these labels was either chemical or enzymatic. Some of these approaches, their limitations, and the detection and visualisation methods they employed are described in Table 1-2 with the relevant references.

Biotin, a B vitamin, is a particularly useful label as it forms high affinity complexes with avidin in addition to being immunocytochemically detectable. The first biotin labelling of nucleic acid sequences was by cross-linking. More general use of biotin as a probe label became possible with the preparation of biotinylated derivatives of UTP and dUTP.

These substrates for nucleic acid polymerases could easily be incorporated enzymatically by means of, for example, a nick translation reaction (Fig.1-5). Though a plethora of indirect probe labelling systems were introduced in the 1980s, enzymatic incorporation of biotin and subsequently of dUTP-conjugated digoxigenin (DIG), a steroid derivative of *Digitalis purpurea*, was the simplest and most enduring (Fig.1-6).

Fluorescent dyes were amongst the first non-isotopic labels utilised, initially thiosemicarbazide derivatives of FITC (fluorescein isothiocyanate) or TRITC (tetramethyl rhodamine isothiocyanate) incorporated by 3' end labelling of nucleic acid sequences. Immunocytochemical amplification improved the weak signal provided by the single fluorescent molecule on each probe fragment. More efficient fluorescent labelling was later provided by enzymatic incorporation of FITC derivatives of dUTP (Fig.1-6).

1.5.2.2 Detection Methods

These non-isotopic labels were variously detected indirectly by antibody-associated chromogenic enzyme reactions, colloidal gold with silver amplification, or fluorescence. As mentioned above, biotin could be detected immunocytochemically or by avidin conjugated to an enzyme or fluorochrome. Fluorescent labels were either visualised directly where probes were large, complex or repetitive target sequences so producing signals of adequate strength, or amplified using immunofluorescence. Direct detection reduces processing time and immunocytochemically-derived background signals, and removes potential for cross-reactivity of antibodies in different layers. Also, as the stoichiometry of indirect detection is more complex, direct detection represents a more appropriate approach for sequence copy number quantitation.

LABEL	LABELLING METHOD	DETECTION	VISUALISATION	REFERENCE
Biotin (B-vitamin)	cross-linking via cytochrome c	avidin coated microspheres or avidin ferritin	electron microscopy	Manning et al 1975
	enzymatic (e.g. nick translation) incorporation of biotinylated UTP or dUTP	label incorporation assessed by avidin- Sepharose retention or immunoprecipitation immunoperoxidase or immunofluorescence	bright field microscopy or fluorescence microscopy	Langer et al 1981
		immunoperoxidase or immunofluorescence	bright field or fluorescence microscopy	Langer-Safer et al 1982 application to localisation of nucleic acid sequences Manuelidis et al 1982 application to nucleic acid sequence localisation in mammalian cells
		immunoperoxidase or immunofluorescence	bright field or fluorescence microscopy	Hopman et al 1986b
		immunoperoxidase or colloidal gold amplified by silver precipitation	bright field microscopy	Bhatt et al 1986
		avidin-conjugated FITC and immunofluorescence	fluorescence microscopy	Pinkel et al 1986
		chromogenic detection via streptavidin alkaline phosphatase	bright field and phase contrast microscopy	Garson et al 1987
FITC or TRITC	3' end labelling with thiosemicarbazide derivatives of FITC or TRITC	direct sensitivity low - only one FITC molecule per probe fragment	fluorescence microscopy	Bauman et al 1980, 1981a,b,d
FITC-dUTP	enzymatic - nick translation	immunocytochemical amplification direct and immunofluorescence amplification	fluorescence microscopy fluorescence microscopy	Bauman et al 1981c Weigant et al 1991
DNP-dATP 8-(2,4 dinitrophenyl) 2- β - aminoethyl) amino- adenosine 5' triphosphate	enzymatic incorporation 3' end labelling or nick translation	immunocytochemical amplification chromogenic detection antibodies not readily available (Morimoto et al 1987)	bright field microscopy	Vincent et al 1982
DNP DNP-dUTP	chemical incorporation enzymatic - nick translation and PCR	immunoperoxidase immunofluorescence	bright field microscopy fluorescence microscopy	Shroyer and Nakane 1983 Reid et al 1992

Table 1-2 A selection of probe labels, detection and visualisation methods (continued overleaf).

AAF N-acetoxy-2 acetylaminofluorene	chemical incorporation; <i>carcinogen-not recommended for routine use</i>	immunofluorescent amplification	fluorescence microscopy	Landegent et al 1984
		immunoperoxidase amplification	reflection contrast microscopy	Landegent et al 1985a
Mercury	chemical incorporation in pyrimidines <i>assessment on mercurated poly(U) with Sepharose-bound poly(A)</i>	chemical detection of mercurated pyrimidine residues by binding to the sulphhydryl group of a hapten-conjugated ligand + immunofluorescent detection of the (trinitrophenyl) hapten group as above; extended to biotinyl and fluorescein haptens groups; antibodies not readily available	fluorescence microscopy	Bauman et al 1983
	chemical incorporation <i>extremely toxic reagents</i>		bright field microscopy fluorescence microscopy	Hopman et al 1986a <i>on microscope preparations</i>
Sulphonate	chemical incorporation of a sulphone group in cytosine	immunoperoxidase detection of sulphonated cytidine involving amplification with biotinylated second antibody	bright field microscopy <i>haematoxylin counterstain</i>	Morimoto et al 1987
Digoxigenin-dUTP	enzymatic - random priming	immunocytochemical conjugation to alkaline phosphatase	bright field microscopy	Heiles et al 1988 <i>Vernole 1990 ISH to chromosomes</i>
	enzymatic - nick translation and PCR	chromogenic detection immunofluorescence	fluorescence microscopy	Reid et al 1992

Table 1-2 (continued) A selection of probe labels, detection and visualisation methods.

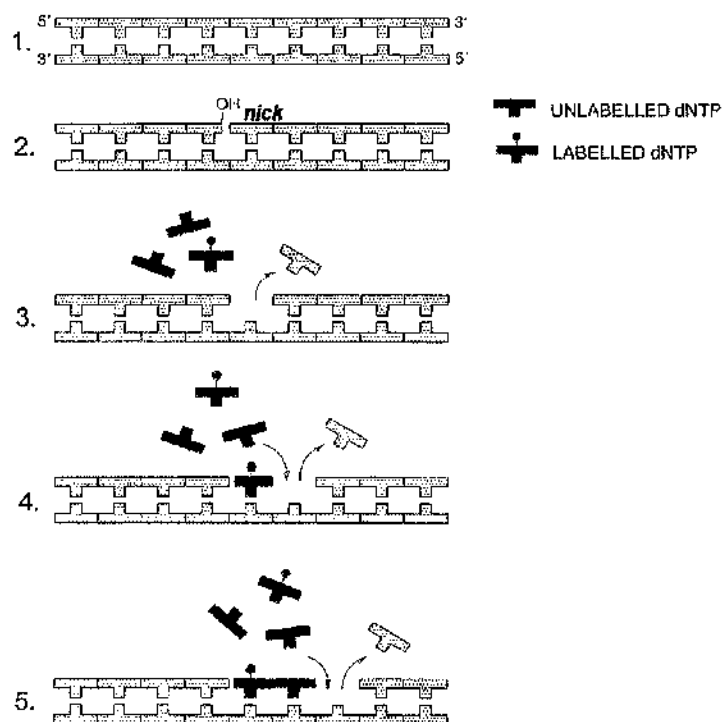


Fig.1-5 The nick translation reaction. Adapted from Leitch et al (1994).

1 and 2. Deoxyribonuclease I (DNase I) introduces single strand breaks ('nicks') to expose free 3'-OH groups.

3. The 5' to 3' exonuclease activity of DNA polymerase I removes nucleotides at the 5'-phosphate side of the nick.

4 and 5. DNA Polymerase I simultaneously synthesises DNA complementary to the intact strand in the 5' to 3' direction using the 3'-OH termini of the nicks as primer. Labelled and unlabelled nucleotides in the solution are incorporated in the newly synthesised strand.

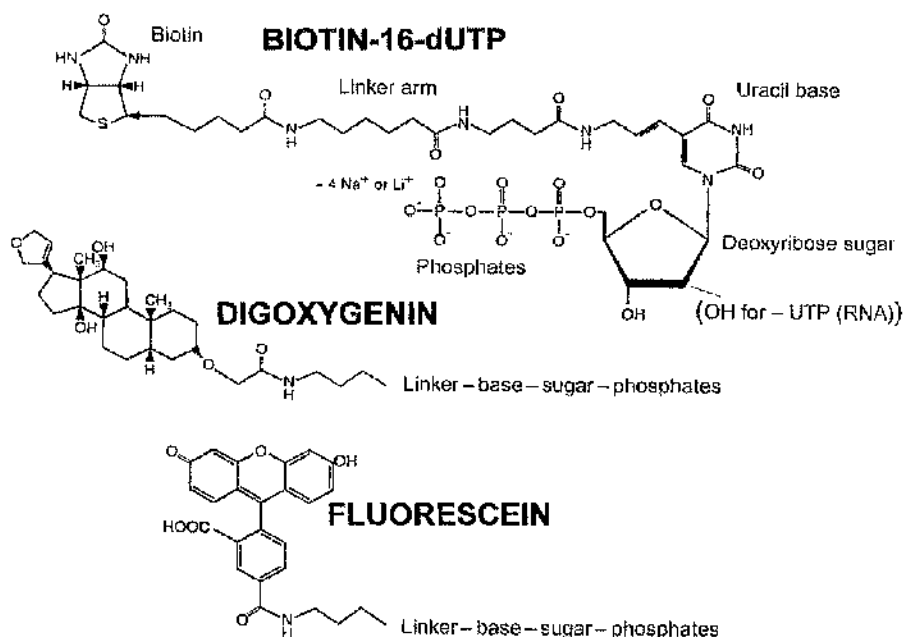


Fig.1-6 Chemical structures of the modified nucleotides most commonly used in non-isotopic ISH.

1.5.2.3 The Limit of Sensitivity of Non-isotopic Methods

The sensitivity of an ISH process is defined as the smallest target detectable. Non-isotopic methods were initially found to be less sensitive than isotopic ISH and restricted to the detection of repetitive sequences (Manuelidis et al 1982) and relatively large single copy sequences of around 20-40kb. A collection of short cosmid-derived subclones of the target sequence, total length 22.3kb, was employed to localise the human thyroglobulin gene (Landegent et al 1985b), and a cosmid probe (insert 35-45kb), because it had few cross-hybridising repeats, was able to be used in mapping to 4p16 a sequence closely linked to the Huntington disease locus (Landegent et al 1986). It was the use of whole plasmid (exploiting vector sequence cross hybridisation) and three layer detection for signal amplification which allowed the sensitivity of avidin-conjugated alkaline phosphatase detection of biotinylated probes to match that of isotopic ISH (Garson et al 1987, Morrison et al 1990). Sensitivity to probes of around 1kb was also demonstrated for amplified immunofluorescent detection of biotinylated probes (Cherif et al 1989, Murphy et al 1993). Non-isotopic ISH using biotin label and immunofluorescence was even claimed to be more efficient than isotopic ISH on comparison of mapping results with chromosome 11q-specific plasmid probes of size range 1.0-3.2kb (Fan et al 1990), although different plasmids were used in the isotopic and non-isotopic studies. Radiolabelled signals were detected in only 20% of cells, whereas fluorescent signals were present in 70% and against much lower background.

1.5.2.4 Factors Affecting the Sensitivity of Non-isotopic ISH

The 'stringency' of hybridisation and post-hybridisation wash conditions determines whether a probe binds specifically to its complementary sequence or nonspecifically, and whether it remains annealed once bound. Stringency is a result of the temperature of incubation and the concentrations of salt and helix-destabilising molecules (e.g. formamide) in hybridisation buffer and wash solutions. Lower incubation temperatures, lower formamide and higher salt concentrations reduce stringency, increasing probe binding and therefore sensitivity while possibly decreasing specificity. Other variables affecting ISH sensitivity are listed below.

- The efficiency of probe labelling. Methods, such as random priming, which can effect higher incorporation of label may provide improved signal intensity (Cherif et al 1989).

- As mentioned above, the presence of vector sequences in the probe may result in signal amplification (Garson et al 1987), though this effect has been questioned (Cherif et al 1989).
- Use of multi-layer detection. Amplification by applying multiple layers of fluorophore immunologically is a controllable feature of fluorescence ISH (FISH) which can optimise signal to background ratio. Signal intensity can be multiplied six times at each amplification (Pinkel et al 1986).
- Use of tyramide amplification (TSA). Increase in sensitivity can follow the use of biotin or fluor labelled tyramides in the indirect fluorescence detection of biotin or DIG labelled probes (Raap et al 1995). In this process, peroxidase is bound to the hybridised probe then tyramide conjugates, which are peroxidase substrates, generate many biotin or fluor molecules at the ISH site. TSA does, however, reduce signal resolution.
- The microscope must be adequately and homogeneously illuminated. FISH requires the appropriate optical filter combinations and use of a high numerical aperture (NA) objective specified for fluorescence e.g. x100, NA 1.4.
- The use of digital imaging (discussed in section 1.5.2.6).
- The amount of repetitive sequence in the probe can affect the apparent sensitivity of a hybridisation as these sequences may require to be blocked (in chromosomal *in situ* suppression, see section 1.5.2.8), reducing the hybridisation efficiency relative to a similarly-sized probe with less repetitive content (Landegent et al 1986).
- Chromosome preparation critically affects penetrance therefore a wide variety of slide pretreatments have been recommended, e.g. pepsin, proteinase K, collagenase, RNase (Leitch et al 1994). Pretreatment is particularly critical with histological sections, uncultured amniocytes or tissue imprints.
- Presence or absence of non-competitive carrier DNA (such as sonicated salmon sperm DNA) and/or dextran sulphate in hybridisation buffer (Harper and Saunders 1981, Manuelidis 1985, discussed in Malcolm et al 1986).
- Hybridisation time and probe concentration. Optimal concentration varies between probes and probe types as does hybridisation time, which is usually 2-24 hours. Raising concentration 5-10 fold may considerably reduce this period (Ward et al 1995).
- Concentration of competitor DNA (Ward et al 1995, section 1.5.2.8).
- Size of hybridised probe fragments. 200-400bp is optimal for ISH (Trask 1991).

As hybridisation efficiency tends to relate to target size, the increasing availability of larger cloned sequences for many applications made ISH sensitivity somewhat less critical.

1.5.2.5 Hybridisation Efficiency Improvement with Larger Probes and CISS

Probes containing 2kb of single copy sequence generally detected a maximum of 40-50% of all target sites and the discrimination of weak specific signals against background required statistical evaluation following the lengthy analysis of many metaphases (Lichter and Cremer 1992).

The availability of larger cloned sequences and, crucially, the chromosomal *in situ* suppression (CISS) method (as described in section 1.5.2.8) for blocking cross-hybridising repetitive sequences in these probes enabled production of larger, more reliable signals (Landegent et al 1987, Lichter et al 1988, Cremer et al 1988, Pinkel et al 1988). A signal is usually produced in at least 80% of the complementary chromosome sites when cosmid (40kb) or YAC clones (100kb-1Mb) are used as probes, and contiguous probe sequences can further improve signal intensity (Landegent et al 1987). This allowed the examination of fewer metaphases, obviated statistical analysis, and further simplified and accelerated the process of ISH.

Through optimising hybridisation efficiency and sensitivity direct visualisation of directly fluorophore-labelled probe, with the concomitant benefit of reduction in processing time and background signal, became increasingly feasible.

1.5.2.6 Visualisation - Microscopy and Recording

Non-isotopic ISH methods have employed a range of visualisation methods (examples in Table 1-2). Visualisation of fluorescent ISH sites is generally by epifluorescence (most frequently using mercury vapour lamps) with appropriate objectives and filter sets for each fluor (Fig.1-7, Table 2-1).

The impermanent nature of FISH preparations made recording of the result essential. However the long exposure times required by conventional cameras to photograph the low light intensity fluorescent signal using colour film often resulted in poor image quality and signal fading. This was overcome with the adoption of electronic imaging systems. The most sensitive, capable of photon counting over long integration times, was the cooled charge coupled device (CCD) camera (described in Aikens et al 1989). This allowed detection of signals invisible to the microscopist's eye and further facilitated the use of directly-labelled fluorescent probes, improving signal:noise ratio (Weigant et al 1991). Cooling of the chip suppressed background noise from dark current (thermal generation of electrons during long exposures).

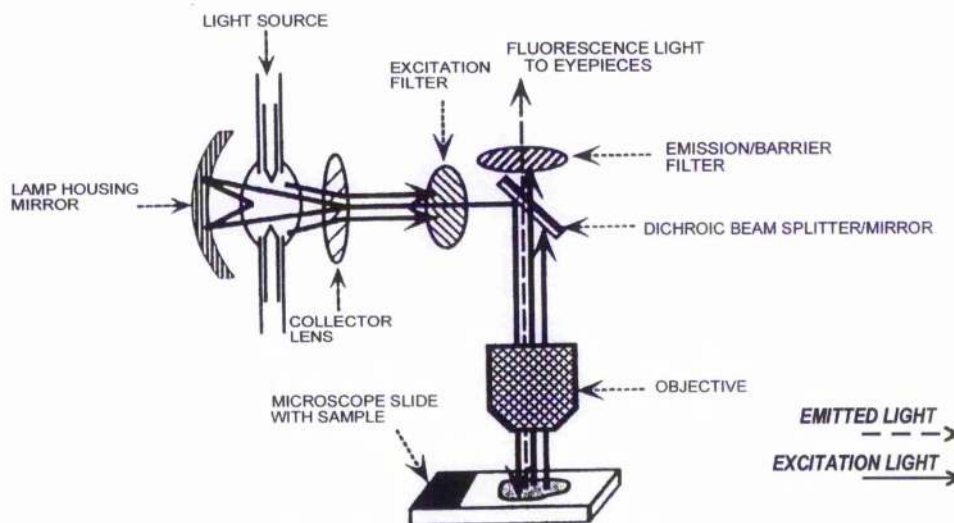


Fig.1-7 The key features of the optics of an epifluorescence microscope.

Adapted from Notes on Fluorescence Microscopy (Vysis Inc).

The mercury vapour light source emits a range of light wavelengths, together appearing white, which pass through the condenser (collector) lenses. The excitation filter then blocks all wavelengths except those that excite the fluor to be visualised. The dichroic beam splitter or mirror reflects some shorter wavelengths (those that excite the fluor) on to the specimen and transmits longer wavelengths (emitted by the fluor) returning from the specimen. A barrier filter then blocks transmission of all wavelengths except those emitted by the fluor, and removes stray reflected light from autofluorescence, before the light reaches the eyepiece lens or camera.

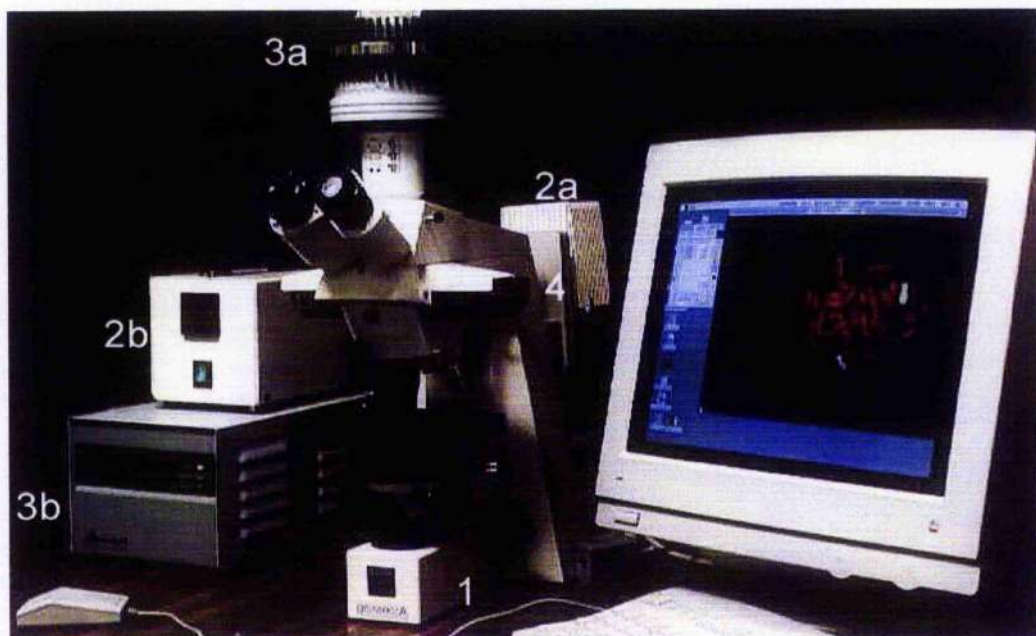


Fig.1-8 Digital imaging system (Digital Scientific, Cambridge).

1: Zeiss Axioskop Microscope

2: Fluorescence light source (a) and control box (b)

3: Photometrics cooled charged coupled device camera (a) and control box (b)

4: Ludl filter wheel

In addition to high sensitivity (digital imaging could result in 30 fold improvement, Weigant et al 1991), CCD cameras also provided high spacial resolution and linearity with wide dynamic range, ideal for low light level FISH and quantitation.

Colour CCD cameras initially had lower sensitivity and resolution and were disadvantaged for advanced applications of ratio imaging (section 1.5.2.7) and quantitative fluorescence microscopy, so black and white cameras became standard. These generate grey scale images separately for each fluor (e.g. signal and chromosome counterstain) which are then pseudocoloured and electronically overlaid. Moving between different filters, just as when using multiple exposures of colour film in conventional photomicrography, could however cause a change in the optical axis leading to incorrect registration of superimposed images. This problem was solved by using a fixed dichroic mirror with a fixed newly developed multiple bandpass emission filter (to allow the simultaneous emission of signals of two or more wavelengths) in combination with selectable excitation filters on a separate motorised wheel which could be computer-controlled (Fig.1-8). Digital imaging also simplifies storage of FISH data and allows image processing, including enhancement of signal and of DAPI bands to aid chromosome identification.

1.5.2.7 Multiple Target FISH

Simultaneous detection of more than one target sequence saves time and material. Fluorescence detection, directly or indirectly, has the advantage that multiple, differently-labelled probes may easily be used together, each detected by a different fluorochrome concurrently (Nederlof et al 1989, Wiegant et al 1991). It was primarily this feature, and the recognition that fluorescent signal offered the best prospect for automated chromosome analysis, that led to the expansion in use of fluorescence detection. Mixtures of probe labels (combinatorial labelling) can be used to provide more detection colours than there are available fluors, thereby extending the number of simultaneously detectable FISH targets (Nederlof et al 1990). The number of targets which can be distinguished by combinatorial labelling is $2^n - 1$ where n is the number of fluorochromes. Hence a digital imaging system, with its ability to pseudocolour and merge images, could be used to distinguish up to seven probes using only three labels (e.g. biotin, DIG, DNP-dUTP) and fluors (Reid et al 1992, Fig.1-9). Conventional photography, requiring multiple exposures of colour film, could not adequately display and resolve images from these combinatorially labelled probes. dUTP derivatives of biotin, DIG, DNP and FITC were the most practical labels to combine because of the identical labelling formats and ISH conditions. By simply mixing

haptens dUTPs in the nick translation it is possible to label one probe with multiple reporters (and detect it with multiple fluorors). Technological improvements enabled differential labelling, and hence colour karyotyping, of all 24 human chromosomes by approaches known as SKY (spectral karyotyping, Schrock et al 1996) and M-FISH (multiplex FISH, Speicher et al 1996).

Ratio-labelling, the use of different proportions of reporters on probes, further extended the multiplicity of combinatorial labelling, initially allowing twelve colours to be determined using 3 labels (Dauwerse et al 1992). Fluorescence ratio imaging of double-labelled probes in multiple ISH was shown to be feasible (Nederlof et al 1992) but ratio-labelling failed to become widely applied due to the demanding technical aspects associated with accurate determination of the relative amounts of each label staining each target.

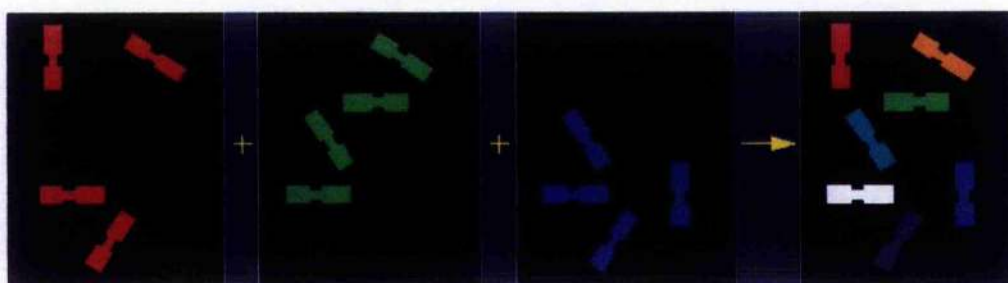


Fig.1-9 Schematic representation of 7-colour FISH using probes combinatorially labelled with three reporter molecules. Adapted from an image kindly supplied by Vysis Inc.

1.5.2.8 Probe Types and Generation

Probes used for ISH can be categorised as repetitive target sequence probes, unique target sequence probes and composite probes, which allow specific 'painting' of individual chromosomes or chromosome regions.

Repetitive Target Sequence Probes

Unique or low copy sequences, which include structural genes with their related regulatory sequences, represent around 50-70% of total human DNA (Clark and Wall 1996). The remainder is repetitive DNA. This is classed as moderately repetitive DNA, which has several hundred sequence copies and includes the genes encoding transfer RNA, ribosomal RNA and histones, and highly repetitive DNA, in tens of thousands to one million copies, which comprises ~20% of the genome and may have a role in chromosome pairing, alignment and recombination. Highly repetitive sequences can be arranged as tandem repeats or interspersed throughout the genome as Long or Short Interspersed Elements (LINEs or SINEs). The most abundant SINE is the Alu family,

consisting of several hundred thousand copies of a 280bp sequence which is flanked by recognition sites for the enzyme Alu I. The average frequency is one copy per 3kb. LINEs are over 500bp long. The L1 (LINE 1) or Kpn family consists of 50,000-100,000 copies found on average every 8kb. Alu sequences predominate in Giemsa-light and L1 in Giemsa-dark bands.

Highly reiterated tandem repeats are known as satellite DNA from the minor bands produced, separate to bulk DNA, by satellite I, II and III DNA on equilibrium centrifugation in a caesium chloride density gradient. Depending on the average size of the repeat unit arrays, this non-coding DNA is sub-grouped as satellite (100kb-several Mb), mainly found at centromeres, minisatellite (0.15-20kb) which includes telomeric and hypervariable families, and microsatellite (under 150bp) which is dispersed throughout chromosomes. Alpha satellite DNA is, like ALU sequences, primate-specific. It constitutes the bulk of the centromeric heterochromatin (a few hundred kb-several Mb), and accounts for ~3-5% of the DNA of each chromosome. Its basic unit is a 171 bp monomer. Divergence in higher order organisation has resulted in alpha satellite repeats on most human chromosomes being chromosome-specific (reviewed in Willard and Waye 1987, Choo et al 1991). Probes recognising alphoid sequences have strong but discrete signal which makes them useful for chromosome copy number determination in metaphase and interphase cells. Other repetitive sequences used for ISH analysis include satellite II and III probes from proximal Yp and Yq, 1, 9, and 16 heterochromatic regions, beta satellite sequences from the heterochromatic regions of 1, 9, Y and acrocentric chromosomes, and satellite III heterochromatin from the short arm of 15. Usually a repetitive probe consists of cloned DNA containing one or a few of the repeat elements (often in a plasmid vector) but they can be generated from genomic DNA by polymerase chain reaction (PCR) amplification (Dunham et al 1992).

Unique Target Sequence Probes

These detect specific sequences present in only one copy in the genome. As previously discussed, ISH with unique sequences was initially confined to the use of short stretches of DNA, often in plasmid or bacteriophage lambda vectors (incorporating inserts of up to 15kb, Harper and Saunders 1981), or to cDNA sequences (Malcolm et al 1981). Vectors with larger insert sizes, such as cosmids (~ 40kb, Collins and Hohn 1978), potentially offered increased target size and reliability of ISH, but their longer inserts contained LINEs and SINES which cross-hybridised causing high background signal. They became usable as ISH probes with the crucial introduction of chromosomal *in situ* suppression (CISS) hybridisation (Landegent et al 1987), an approach earlier investigated during the first mapping of a single copy gene (Harper and Saunders 1981). It involved co-

hybridisation of unlabelled competitor DNA (total genomic DNA or the Cot1 fraction enriched for highly repetitive sequences) to saturate the repetitive probe elements leaving the unique sequences free to hybridise to the target loci (Fig.1-10).

Even longer probe inserts (up to 2Mb) became available with the introduction of yeast and bacterial artificial chromosomes (YACs, BACs), bacteriophage P1 clones and P1-derived vectors (PACs), as reviewed in Monaco and Larin (1994).

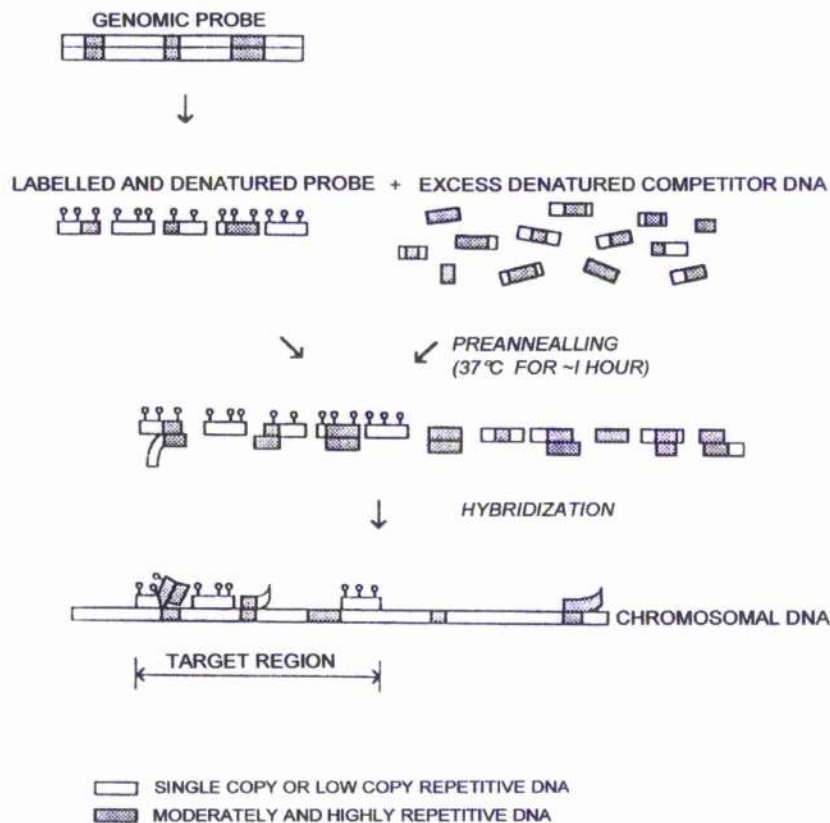


Fig.1-10 Chromosomal *in situ* suppression (CISS) hybridisation. Adapted from Lichter and Cremer (1992).

Chromosome Paint Probes

Chromosome paints are collections of sequences spanning the length or a specific section of a target chromosome. Chromosome painting was developed at Lawrence Livermore National Laboratories (Pinkel et al 1988) and Yale University (Cremer et al 1988, Lichter et al 1988) using cloned DNA libraries derived from flow-sorted human chromosomes. As with large insert probes, cross-hybridising elements were blocked with CISS hybridisation. These early bacteriophage libraries had high proportions of vector DNA but re-cloning in plasmid increased insert to vector ratio (to 0.5 from 0.1 in phage), reducing background hybridisation (Fuscoe et al 1989, Collins et al 1991).

Paints were also produced without cloning from human-rodent hybrids containing single human chromosomes (Kievits et al 1990) and made more sensitive by differentially amplifying the human sequences by interspersed repetitive sequence (IRS) PCR with Alu or L1 primers (Lengauer et al 1990). PCR with degenerate oligonucleotide primers (DOP), which theoretically prime every 4kb, was used on either small numbers of flow-sorted or microdissected chromosomes to rapidly generate paints with further improved signal to noise ratio and target coverage (Telenius et al 1992, Meltzer et al 1992). Labels such as biotin can conveniently be incorporated during such PCR. With microdissection, specific chromosome arm or band probes could also be produced (Guan et al 1996). Depending on degree of coverage, background noise and target chromosome condensation, paints can resolve approximately 5Mb of DNA (Rosenberg et al 1992).

1.6 The role of ISH in Human Gene Mapping

ISH has become a versatile tool in many research disciplines including radiation biology (Cremer et al 1990), evolutionary cytogenetics (Weinberg et al 1990), meiotic studies (Goldman and Hulten 1992), nuclear topography (Manuelidis 1985, Lichter et al 1988) and the organisation and replication of human DNA (Korenberg and Rykowski 1988, Rosenberg et al 1995). The first application of ISH, however, was in sequence mapping. The major reason for mapping human genes is to improve our basic understanding of the human genome and, through this, the role of particular genes in health and disease. The two main strategies in sequence localisation are linkage and physical mapping.

1.6.1 Linkage Analysis and Early Mapping Studies

In family linkage studies the chromosomal location of one trait is known and the frequency of rearrangement between this and a second sequence or trait is a measure of the linkage between these (0%=tight linkage, 50%=none). Tight linkage suggests two sequences co-localise to the same chromosomal region.

Prior to the cloning era protein variants, such as blood groups and serum protein markers, and chromosomal heteromorphisms (e.g. 1qh+, 9ph) were used as polymorphic marker traits for such studies. Other early mapping approaches involved the correlation of gene product with the presence of a chromosomal region by exploiting gross chromosomal duplications and deficiencies (gene dosage and exclusion mapping studies) and translocations in somatic cell hybrids (reviewed in Ferguson-Smith and Aitken 1982).

Recombinant DNA technology initially provided hybridisation, both *in situ* and to Southern blots following restriction enzyme digestion (reviewed in Davies 1981), then, in the late 1980s, PCR methods for dosage and somatic cell hybrid studies (example in Abbott et al 1989). More highly polymorphic markers for linkage analysis became available, in the form of restriction fragment length polymorphisms (RFLPs) and variable number tandem repeats (VNTRs, comprising minisatellite and microsatellite repeats). Microsatellite markers (short tandem repeat polymorphisms, STRPs, of di, tri or tetranucleotides) have been particularly useful, being abundant, evenly dispersed in the genome, highly informative and easy to type (discussed in McConkey 1993).

In 1989 the international research community established the Human Genome Organization (HUGO) to promote collaboration and coordinate human genome mapping. The same reference families were used by different research groups for all linkage studies and their data combined to produce integrated maps. This effort was expedited by adoption of a 'factory' approach, only possible in dedicated laboratories, using large scale automation and computerised statistical linkage analysis on polymorphic repeat markers.

By 1994 a map of 5840 loci provided a marker density of one per 0.7cM (Murray et al 1994). Marker locations were submitted to databases, primarily the Genome Database (<http://www.gdb.org>), making this information widely available electronically. The expanding linkage map was to provide the framework for construction of a physical map, allowing sequences to be mapped to intervals between the PCR-based markers.

1.6.2 Physical Mapping

Physical mapping aims at the isolation, chromosome localisation, ordering and sequencing of contiguous cloned pieces of human DNA, ultimately covering all areas of all chromosomes. The ordering of sets of sequences requires a hierarchy of stages at different levels of resolution.

Physical mapping using recombinant DNA technology began in the 1970s in a sporadic fashion, with both restriction mapping of DNA in somatic cell hybrids and isotopic ISH to chromosomes of repetitive, and later unique, sequences as these were isolated by independent investigators and ISH sensitivity improved (Davies 1981).

Systematic physical mapping was made feasible by the introduction of pulsed-field gel electrophoresis (PFGE, Schwartz et al 1983) followed by the crucial development of YACs (Burke et al 1987). PFGE allowed differential separation, and therefore analysis by

molecular hybridisation and construction of long-range restriction maps, of large (50kb-1Mb) DNA molecules. YACs provided a means of cloning such large sequences. Isolation and characterisation of clones from genomic YAC libraries allowed production of coarse contig maps of individual human chromosomes (Cohen et al 1993). Smaller insert vectors could then be used to assemble high-resolution contig maps, a prerequisite for sequencing of whole chromosomes. Partial sequencing of clones reveals unique STS (sequence tagged sites) and ESTs (expressed sequence tags, 200-300bp), and STS/EST maps can be used to help localise other clones according to their STS and EST content. ISH has been involved at different stages of this physical mapping process. For example:

- Confirming the human chromosome content of somatic cell hybrids prior to production of single chromosome YAC libraries from these, and confirming the chromosomal localisation of cloned sequences (Montanaro et al 1991).
- Checking the chromosome-specificity of YACs selected from a genomic library for the preparation of a single chromosome library (Ross et al 1992).
- Ascertaining YAC inserts do not contain artefactually-linked sequences from non-contiguous genomic regions (YAC libraries have a high frequency of chimaeric clones).
- Band localisation of STRPs in YAC clones (Murray et al 1994).
- Ordering clones, as an alternative to PFGE (Lebo et al 1992).
- The localisation of ESTs (in human cDNA clones) as an alternative to PCR screening of somatic cell and radiation hybrids (Korenberg et al 1995).

In summary, ISH complements other mapping approaches and is often involved in initial localisation, confirmation, ordering and (as described below) orientation of clones because it offers some advantages over other techniques. It is direct, rapid, gives excellent regional localisation and does not require production and maintenance of regional mapping panels of somatic cell hybrids or flow-sorted chromosomes for dot-blot production. Nor does it depend on availability of polymorphic markers or large pedigrees. Eventually, by integrating linkage and physical maps produced by the Human Genome Mapping Project, it will be possible to find the genes for all diseases and biological functions. However, while awaiting this achievement, it remains useful to map, as they are isolated, sequences that could be candidate genes.

Such specific sequences can be isolated by functional cloning (where the gene product is known so its sequence can be inferred) or positional cloning. In positional cloning a gene must first be mapped, for example through association of a specific chromosomal aberration with abnormal gene function (as in the case of the dystrophin gene, Kunkel et al 1985). The DNA at that locus is then screened for genes by, for example, identification of hypomethylated CpG islands. That the gene of interest has been isolated is confirmed

by finding mutations in patients with the disease or from the distribution and function of the predicted protein.

ISH to chromosomes prepared from cultured lymphocytes, an approach particularly accessible to cytogenetic laboratories, is often the method of choice for mapping functionally cloned sequences or confirming the localisation of sequences obtained by positional cloning.

1.6.3 Mapping DNA Sequences to Chromosomes using ISH

1.6.3.1 Use of Standard Banding Techniques

For mapping data obtained by ISH to chromosomes to be expressed in cytogenetic terms, a banding method must be combined with the hybridisation protocols. Banding can be induced by essentially standard techniques before or following ISH (reviewed in Lichter and Cremer 1992). Early applications often involved pre-, rather than post-ISH G-banding (with Lipsol or trypsin treatment) to avoid interference of heat denaturation with banding quality (Malcolm et al 1986, Garson et al 1987, Klever et al 1991). There was no risk of banding failing following ISH and, when statistical analysis of signal distribution was necessary, it removed bias in post-ISH metaphase selection. However it required photography of banded chromosomes, destaining, then re-location of previously photographed metaphases post hybridisation for signal placement on bands.

Banding after ISH, e.g. with Quinacrine, Hoechst 33258 or DAPI, was more convenient and less time consuming, but the band quality was variable (Lichter and Cremer 1992). R-banding could be obtained post-ISH by a rather complex process involving BrdU incorporation during cell culture then, usually, Hoechst staining and UV irradiation (Cherif et al 1990). Counterstaining was with propidium iodide (PI), PI plus DAPI, or, for permanent replication banding following chromogenic enzymatic detection, by Giemsa. Photography was simplified and the ease and precision of mapping probes to bands improved if banding could be viewed together with probe signal without a change of optical filter. To detect FISH signals and bands simultaneously, counterstain and probe label had to produce distinct colours with the same excitation filter (PI stain and FITC label both excite at 450-490nm but emit in red and green respectively). Double, triple and even quad bandpass optical filters now allow simultaneous visualisation of multiple fluor.

1.6.3.2 Chromosome and Regional Identification Without an Orthodox Banding Protocol

Specific chromosomes are easily identified without banding by simultaneously hybridising a marker probe for the chromosome of interest (e.g. an alphoid sequence). Though co-hybridisation to chromosome-specific 'bar codes' (combinations of probes which generate staining patterns for regional identification) was proposed as a way to increase resolution (Lengauer et al 1993) this was not routinely adopted. Incomplete suppression of Alu sequences can provide an R-banded background hybridisation pattern (Francke 1995), and FISH with Alu or LINE sequences produces R- or G-bands respectively (Korenberg and Rykowski 1988, Lichter et al 1990, Baldini and Ward 1991).

The most convenient alternative banding approach is provided by digital imaging software which enables enhancement and contrast reversal of DAPI counterstain Q-banding, resulting in a low-resolution G-banding.

Accurate mapping without bands can be achieved by mapping with respect to a reference point on the chromosome. The short arm telomere has been used as reference and distance to the probe signal expressed as a fraction of the chromosome length (the FLpter value, Lichter et al 1990). This approach can be useful for mapping the relative localisation of multiple probes but chromosome length polymorphisms and differential homologue condensation may affect FLpter values. Also ISCN banding ideograms are not normalised relative to the fractional length so it is not appropriate to use this measure to indirectly assign a signal to a band. Other reference points potentially useful for mapping and ordering sequences include fragile sites and regions of structural abnormality such as translocation breakpoints (Ferguson-Smith and Aitken, 1982).

1.6.4 High Resolution Mapping and Ordering of Sequences

As larger signals are more likely to co-hybridise, higher resolution ISH mapping and the ordering of more tightly linked markers is more readily achieved with smaller sequences, e.g. bacteriophage λ rather than YAC probes (Knoll et al 1993). The fluorescent signal produced by a cosmid probe on metaphase chromosomes is usually $< 0.3\mu\text{m}$ in diameter. The other factor affecting resolution is chromatin condensation. Banded metaphase chromosomes provide the coarsest ISH mapping targets, variable condensation of bands and slight variations in size and position of ISH sites limiting resolution to 5Mb (Trask 1991), or 3Mb when mapping relative to the telomere (Lichter et al 1990). Resolution can be improved to $\sim 1\text{Mb}$ by co-localisation with a differently-labelled probe of known location.

Chromosome packaging may distort the linear order of adjacent sequences - correct orientation was seen in only 59-70% of chromatids hybridised to two cosmids even with 2-3Mb separation (Trask et al 1991). This can necessitate scoring of multiple chromatids to derive probe order with statistical confidence. Ordering may be particularly problematic at the telomeres (Trask et al 1991).

For increased ISH mapping resolution, less condensed chromatin preparations must be targeted (Table 1-3). To this end, cytogenetic preparations can be enriched for prophase or prometaphase chromosome spreads by synchronised cell culture (Yunis 1976), meiotic chromosomes can be used (Jhanwar et al 1983, Bello et al 1989) or nuclei can be fused with mitotic cells leading to 'premature chromosome condensation' (Sperling and Rao 1974). Cytocentrifugation of unfixed, hypotonically-treated metaphase preparations produces stretched chromosomes which allow rapid ordering of clones $\geq 200\text{kb}$ apart, though variable regional stretching permits only estimation of sequence distance (Haaf and Ward 1994).

Even higher resolution is achieved using G_1 nuclei (Lawrence et al 1988, Trask et al 1989, 1991). Interphase mapping has at least ten times the sequence ordering resolution of dual colour FISH to metaphase chromosomes (25 or 50-100kb versus 1Mb). A set of probes is ordered by comparing the distances between differentially labelled pairs of probes (Trask et al 1989). Within 25-2000kb there is a linear relationship between the genetic interval and mean square of the distance between two signals (Yokota et al 1995a). As an alternative to distance measurements, multiple probes labelled differentially can be hybridised and ordering performed by determining which probe order occurs with highest frequency (Trask et al 1991).

Above 1-2Mb distance, packaging of the interphase chromatin begins to affect signal distance and probe order, though alkaline borate treatment can expand the range of interphase FISH mapping to 4Mb (Yokota et al 1995b). Extension of mapping resolution, to 20kb, was achievable by FISH to decondensed chromatin of sperm pronuclei (Brandriff et al 1991) but the method was laborious and required specialised expertise.

Fortunately more direct means of obtaining decondensed DNA were developed. The term fiber FISH came to be applied to several methods involving FISH to extended DNA stretched on a glass slide, including free chromatin, halo preparations, DIRVISH, and molecular combing (references in Table 1-3). Chromatin organisation is loosened by alkali, DNA relaxation reagents such as topoisomerase II inhibitor m-AMSA (both Heng et al 1992), high salt (Weigant et al 1992) or detergent (Para and Windle 1993) treatment of cell preparations. Fibres can also be prepared from DNA fragments in agarose blocks following PFGE (Heiskanen et al 1994). In molecular combing, DNA in solution is

linearised by allowing one end of the DNA molecule to bind to a slide, then the solution is coverslipped and DNA stretched by the hydrodynamic action of the receding meniscus. This yields high fibre density with minimal shearing and an average fibre condensation of 2.3kb/um (Weier et al 1995). Relaxed duplex DNA has 2.9kb/um.

Fiber FISH is applicable to the determination of physical distances between signals by comparison to the known length of one probe (Para and Windle 1993) though direct conversion of measurement to kilobases is possible if stretching is uniform, as in molecular combing (Weier et al 1995). Fibres are generally used for resolution of overlapping and adjacent clones 1-500kb apart (Florijn et al 1995, Weier et al 1995) though longer ISH distances have been reported (700kb, Para and Windle 1993). ISH to genomic DNA fibres allows sequence ordering and structural analysis (Haaf and Ward 1994), ordering of contig elements (Florijn et al 1995), and study of rearrangements in disease genes such as the detection and measurement of micro-rearrangement or deletion (Florijn et al 1995, Heiskanen et al 1995a, Weber-Hall et al 1996). The alternative, PFGE, can be problematic and lengthy. With probes from the 5' and 3' ends, sequence transcriptional orientation is determinable with respect to flanking regions (Heiskanen et al 1995b, Nishio et al 1996). FISH to fibres from large DNA clones, e.g. YACs, provides high resolution information on insert integrity and stability, positioning of smaller clones within the YACs, and replication origin data (Weier et al 1995, Rosenberg et al 1995).

TARGET MATERIAL	RESOLUTION (kb)	ADVANTAGES(+) /DISADVANTAGES(-)	REFERENCES
metaphase chromosomes	>1000	+tel-cen orientation +mapping to specific band -Low resolution	Weigant et al 1991
mechanically-stretched chromosomes (x5-x20)	>200	+tel-cen orientation -distance not determinable	Haaf and Ward 1994
interphase nuclei	25/50 to 1000/2000	+distance determinable -no tel-cen orientation	Trask et al 1989, 1991
sperm pronuclei	20 - 800	+distance determinable -no tel-cen orientation -specialised technique	Brandriff et al 1991
free chromatin	20 - >350	+accurate distance determination -no tel-cen orientation	Heng et al 1992
halo-DNA	10 - 200	as above	Weigant et al 1992
DIRVISH fibres	<5 - >700	as above	Para and Windle 1993
molecular combing fibres	as above	as above, with straight, homogeneously stretched, fibres	Bensimon et al 1994

Table 1-3 Mapping resolution obtainable with different ISH targets.

Adapted from Buckle and Kearney (1993) and Heiskanen et al (1996).

1.7 Diagnostic Application of Fluorescence *In Situ* Hybridisation

With the introduction of CISS hybridisation and increasing availability of clinically relevant probes, some from convenient commercial sources, FISH began to be exploited as a diagnostic technique and found application in many areas of clinical cytogenetics.

Visualisation and localisation of specific DNA sequences by FISH is possible in a wide variety of diagnostic material. Apart from the commonly studied metaphase spreads and interphase nuclei of standard cytogenetic preparations, other probed preparations include uncultured amniocytes (Klinger et al 1992), tissue sections (Leong et al 1993, Afify et al 1996), tumour touch imprints (Taylor et al 1994), blood smears (Anastasi et al 1992) marrow smears and core biopsy sections (Taylor et al 1994, Miranda et al 1994) and blastomeres (Delhanty et al 1993). FISH can also be performed on specimens which have already undergone cytogenetic analysis (Klever et al 1991).

In postnatal constitutional cytogenetics, accurate identification of chromosome aberrations by FISH is important for prognosis assessment and, where relevant, facilitating future rapid PND of familial rearrangements. It may also allow eventual identification of clinically significant genes at rearrangement breakpoints. Interphase FISH can provide rapid testing on uncultured lymphocytes, or where culture has failed. In PND, apart from metaphase FISH for the identification or confirmation of abnormalities detected during conventional cytogenetics, interphase FISH has enabled rapid aneuploidy detection. Maternal anxiety and the limited time available for any intervention makes speed essential in PND, and FISH on uncultured amniocytes gives a result in 2 days, instead of the 7-10 days for culture and conventional cytogenetics (Guyot et al 1988, Klinger et al 1992). Multicolour ISH can further expedite analysis (Reid et al 1992, Divane et al 1994).

FISH has also been used in association with developmental techniques designed to achieve non-invasive PND, such as screening foetal cells from maternal blood (reviewed by Steele et al 1996) and transcervical cell sampling (Adinolfi et al 1995). Preimplantation genetic diagnosis (PGD) has involved FISH in sex determination for couples at risk for X-linked disease (Delhanty et al 1993, Griffin et al 1994) and in aneuploidy testing (Munne and Weier 1996).

FISH, in addition to other molecular techniques, is extensively used to address difficulties in cancer cytogenetics. Specifically, cytogenetic preparations from neoplastic material may be highly aneuploid with complex chromosomal abnormalities which FISH can clarify. Alternately, they may not yield analysable metaphases or may have very low mitotic index. Further, some clinically significant abnormalities, such as trisomy 12 in B-CLL, are only present in non-dividing cells (Anastasi et al 1992). Interphase FISH facilitates

analysis of high numbers of dividing and non-dividing cells and detection of abnormalities present in only a small proportion. Its use can avoid the risk of bacterial contamination or overgrowth of sub-populations or stromal cells associated with tumour culture, and ISH identification of chromosome abnormalities in focal, tumour cell areas of tissue sections allows genetic testing to be related to specific tumour histology.

Diagnostic Use of Chromosome Paints

Paints, applied singly or in combination, have a wide range of uses in clinical diagnosis. They are frequently employed, in constitutional as well as malignant cells, in determining the chromosomal origin(s) of structural abnormalities, known as 'markers', which are unidentifiable by banding. Probe choice is often guided by clinical phenotype or marker banding pattern, and multiple hybridisations might be required to fully characterise the abnormality. Used in conjunction with banding, painting can also improve interchromosomal rearrangement breakpoint definition (Cremer et al 1988).

Generation of paint from a flow sorted or microdissected marker for ISH to normal metaphase spreads, reverse painting, has successfully resolved structural chromosomal abnormalities and can reveal unsuspected complexity (Carter et al 1992, Blennow et al 1992). This is one way of removing the need to identify a suitable probe or use a series of probes for any investigation, but it requires specialised expertise and equipment. Small marker chromosomes may contain no, or very small amounts of, euchromatin so may not be identifiable by painting. Other factors affecting the utility of paints include their limited resolution (5-10Mb) and inability to detect small interstitial rearrangements - deletions, duplications or inversions (Rosenberg et al 1992). Also, while translocations have been detected in nuclei using paints (Pinkel et al 1988, Cremer et al 1988), their diagnostic application is generally confined to metaphase analysis, as interphase paint signals are diffuse and difficult to quantitate.

Diagnostic Use of Repetitive Target Sequence Probes

Centromere-specific repetitive target sequence probes singly, or in a systematic series of multicolour combinations, are extremely useful for the identification of small marker chromosomes (Callen et al 1992). They are also widely used as control probes, highlighting target chromosomes to which diagnostic probes are hybridised. Their reliable, discrete signal has made them ideal for a wide range of interphase FISH cancer studies (for example Anastasi et al 1991, 1992, Chen et al 1992, Macoska et al 1993, Micale et al 1993, Wessman et al 1993, Murphy et al 1995, Afify et al 1996) and, particularly because they require only a few hours hybridisation and so provide rapid

chromosome enumeration, PND and PGD. Here chromosome X, Y and 18 scoring usually employs repetitive sequences (Divane et al 1994, Cacheux et al 1994, Munne and Weier 1996). However, centromeric alphoid sequences are heteromorphic and this (Verma and Luke 1992) as well as Robertsonian translocation (Spathas et al 1994) and somatic pairing of centromeric regions, may, rarely, lead to misinterpretation. The closely homologous alphoid sequences on chromosomes 13 and 21, as well as 14 and 22, can not be differentiated by alpha satellite probes, rendering their use for interphase chromosome scoring unreliable (Cacheux et al 1994).

Diagnostic Use of Unique Target Sequence Probes

These also can be used as chromosome identifiers. For example, as no chromosome 21-specific alphoid probe is available, collections of chromosome 21-specific plasmids were used to diagnose trisomy 21 in nuclei (Lichter et al 1988). Later use of cosmids and YACs improved reliability (Klinger et al 1992, Romana et al 1993). A unique sequence probe is also used for chromosome 13 enumeration (Klinger et al 1992).

A particular asset of unique sequence, locus specific probes is the refinement they bring to FISH study of structurally-rearranged chromosomes, including interstitial abnormalities. They allow more accurate definition of breakpoints, especially when fiber FISH is employed (Florijn et al 1995, Weber-Hall et al 1996).

FISH with unique probes can diagnose deletions of clinically significant sequences in tumours, for example retinoblastoma (Kallioniemi et al 1992a), the amplification of oncogenes, such as *erbB2* in breast cancer (Murphy et al 1995) and *N-myc* in neuroblastoma (Leong et al 1993), and determine ploidy (Taylor et al 1994).

Several unique probe combinations have been designed for visualisation of specific clinically significant rearrangements in metaphase and interphase cells from neoplastic lines. One approach is use of locus specific probes on the two chromosomes involved in a rearrangement, the signals from which appear fused if translocation occurs. Targeted rearrangements include PML/RARA fusion in the t(15;17)(q22;q11~21) diagnostic for AML M3 (Mancini et al 1995), AML/ETO fusion on the derived 8 in the t(8;21)(q22;q22) associated with AML M2 (Sacchi et al 1995), and BCR/ABL fusion due to t(9;22)(q34;q11) in a variety of leukaemias (Tkachuk et al 1990). As with all interphase FISH, careful design of controls, as well as result evaluation, is crucial, especially if these procedures are used for minimal residual disease determination.

The application of unique sequence probes to detection of the subtle chromosome abnormalities associated with microdeletion syndromes and idiopathic mental retardation is discussed in detail in the next section.

1.7.1 Detection of Submicroscopic Chromosomal Rearrangements

A particularly valuable attribute of unique sequence probes is their ability to visualise chromosome aberrations which are beyond the resolution of conventional banding techniques and light microscopy. Such aberrations are termed 'cryptic'.

1.7.1.1 Microdeletion and Microduplication Syndromes

In clinical genetics, the term 'syndrome' is used to denote a recognisable pattern of signs or malformations that characterise a particular condition. The resolving power of unique sequence probes is exploited for FISH testing in a variety of syndromes known to be associated with deletion or duplication of specific, often submicroscopic, chromosomal segments (Table 1-4). As not all the features of a syndrome may be manifest in every patient it is often difficult to establish or exclude the diagnosis beyond doubt. Detection of the appropriate segmental aneusomy confirms the diagnosis of these conditions, and may allow earlier diagnosis and institution of appropriate medical management.

SYNDROME	SEGMENTAL ANEUSOMY	REFERENCE	MIM #
Charcot-Marie-Tooth Type 1A	dup(17)(p11.2p11.2)	Lupski et al 1992 Lebo et al 1993	118220
Miller-Dieker	del(17)(p13.3)	Kuwano et al 1991	247200
Prader Willi and Angelman	del(15)(q11~q13q11~q13)	Kuwano et al 1992	176270 105830
Smith-Magenis	del(17)(p11.2p11.2)	Juyal et al 1995	182290
Rubinstein-Taybi	del(16)(p13.3)	Breuning et al 1993	180849
Williams	del(7)(q11.23q11.23)	Ewart et al 1993	194050
Cri du Chat	del(5)(p15.2~p15.3)	Pettenati et al 1994 Gersh et al 1995	5p15.2 5p15.3 123450
Wolf-Hirschhorn	del(4)(p16.3)	Gandelman et al 1992	194190
DiGeorge/ Velocardiofacial/ Conotruncal anomaly face	del(22)(q11.2q11.2)	Lindsay et al 1993 Scambler et al 1992 Burn et al 1993	188400 192430 217095

Table 1-4 Some microdeletion and microduplication disorders detectable by FISH.
MIM #: Mendelian Inheritance in Man database number

FISH to cytogenetic preparations is an efficient method of detecting such segmental aneusomy as it can be informative more often than polymorphic PCR analysis, faster than PFGE, and faster, more informative and reliable than restriction analysis (Lebo et al 1993). The resolution of interphase FISH is required for microduplication detection whereas microdeletions are commonly detected by FISH to metaphase chromosomes.

DiGeorge/velocardiofacial, Wolf-Hirschhorn and Rubinstein-Taybi syndromes were investigated in this study.

1.7.1.1.1 DiGeorge/Velocardiofacial Syndrome

DiGeorge presented his observations on patients with hypoparathyroidism, aplastic thymus and defective cellular immunity to a Society for Pediatric Research meeting in 1965, though thymic aplasia was first noted in association with congenital hypoparathyroidism in 1959 (Lobdell 1959, DiGeorge 1965).

A wide spectrum of clinical features have now come to be associated with DiGeorge syndrome (DGS, MIM 188400), but the predominant components of the classically defined syndrome are cardiac outflow tract defects, aplastic or hypoplastic thymic and parathyroid glands (presenting as deficient cell-mediated immunity and hypocalcaemia) and dysmorphic facial features (Conley et al 1979). The dysmorphism includes hypertelorism with short palpebral fissures, small mouth with short philtrum, broad-based nose with bulbous tip, unusually shaped auricles and micrognathia. Heart defects include type B interrupted aortic arch, persistent truncus arteriosus, right-sided aortic arch and tetralogy of Fallot (TOF).

The conotruncus, thymus and parathyroids are derived from the 3rd and 4th pharyngeal pouches and 1st branchial arch. DGS is believed to be a consequence of a defect in the interaction of these embryonic structures and the cephalic neural crest at 4-6 weeks gestation (Lammer and Opitz 1986) and appears aetiologically heterogeneous. Exposure to teratogens including alcohol (Lammer and Opitz 1986) and to maternal diabetes (Wilson et al 1993) were noted in patients with DGS. The phenotype was also reported in individuals with a variety of cytogenetically detectable chromosomal defects, notably monosomy of 22pter-q11 in cases of unbalanced translocation involving 22 and interstitial deletion of 22q11 (de la Chapelle et al 1981, Greenberg et al 1988).

Molecular, including FISH, investigations demonstrated that 22q11.2 deletion (often submicroscopic) was actually present in the majority of DGS cases (in 33/35 individuals in one study) and provided a map of the commonly deleted region (Carey et al 1990, 1992, Scambler et al 1991, Halford et al 1993a, Lindsay et al 1993).

Deletion of 10p is the only cytogenetic aberration, other than 22q11.2 deletion, repeatedly associated with DGS and related phenotypes (Goodship et al 1994, Daw et al 1996, review and other references in Greenberg et al 1988, Lipson et al 1996). The other chromosome abnormalities reported in patients with DGS features have been isolated cases (Wullich et al 1991, Fukushima et al 1992, van Essen 1993, Lindgren et al 1994,

Siu et al 1996, others reviewed in Greenberg et al 1988) and, as most had no molecular testing, could also have had cryptic deletion at 22q11. All exhibited additional multiple congenital abnormalities but so too do many 22q11 deletion cases.

Shprintzen or velocardiofacial syndrome (VCFS, MIM 192430) is a syndrome of palatal insufficiency, conotruncal cardiac anomalies, minor facial dysmorphology and mild learning difficulties (Shprintzen et al 1978, 1981), first described by Dr Eva Sedlackova (Sedlackova 1955). The degree of clinical overlap with DGS led to the molecular investigation of VCFS patients (using RFLPs, dosage analysis, and some FISH) and demonstration of 22q11 deletion in the majority of cases (Scambler et al 1992 5/5 cases, Driscoll et al 1992 14/15 cases, 1993 41/55 cases, Lindsay et al 1995 44/54 cases). Deletion at 22q11.2 was also reported in patients with conotruncal anomaly face syndrome (CAF or CTHM, MIM 217095), a condition comprising dysmorphic facies, cardiac outflow tract defects and delayed development (Kinouchi et al 1976, Burn et al 1993). This led to realisation that these syndromes have a common aetiology and that assignment of patients to these three syndromic groups may have resulted from ascertainment bias through clinical expertise in either immunology (DGS), craniofacial malformations (VCFS), or cardiology (CAF, VCFS).

Patients initially assigned other diagnoses such as CHARGE association (MIM 214800), Opitz syndrome (MIM 145410) and Cayler syndrome (MIM 125520) have been found to have 22q11 microdeletion (references in Dallapiccola et al 1996), as have patients with isolated features of DGS such as hypoparathyroidism (Scire et al 1994) and cardiac abnormalities. These included individuals in five out of nine tested families with recurrent outflow tract defects. Although two of these families contained individuals with hypocalcaemia, one child was developmentally delayed and, in retrospect, several individuals were assessed as subtly dysmorphic, these results prompted a preliminary study of 40 clinically normal individuals and 40 individuals with isolated TOF, the most common cyanotic congenital heart malformation. With a prevalence of 2.5 to 3.5 per 1000 live births, classic anatomic findings in TOF are large, non-restrictive, anterior, malaligned, ventricular septal defect (VSD) with pulmonic stenosis or atresia and overriding aorta. Chromosome 22q11 microdeletion was found in 2 (5%) of the TOF group and none of the controls (Wilson et al 1992a).

Microdeletion at 22q11 appears to be the most frequent segmental aneusomy. One in 9700 births has 22q11 deletion associated with a typical DG/VCFS phenotype, but the overall prevalence of the deletion is estimated at 1:4000 when including those deletions found in approximately 5% of individuals with non-syndromic congenital heart defects (Tezenas du Montcel et al 1996).

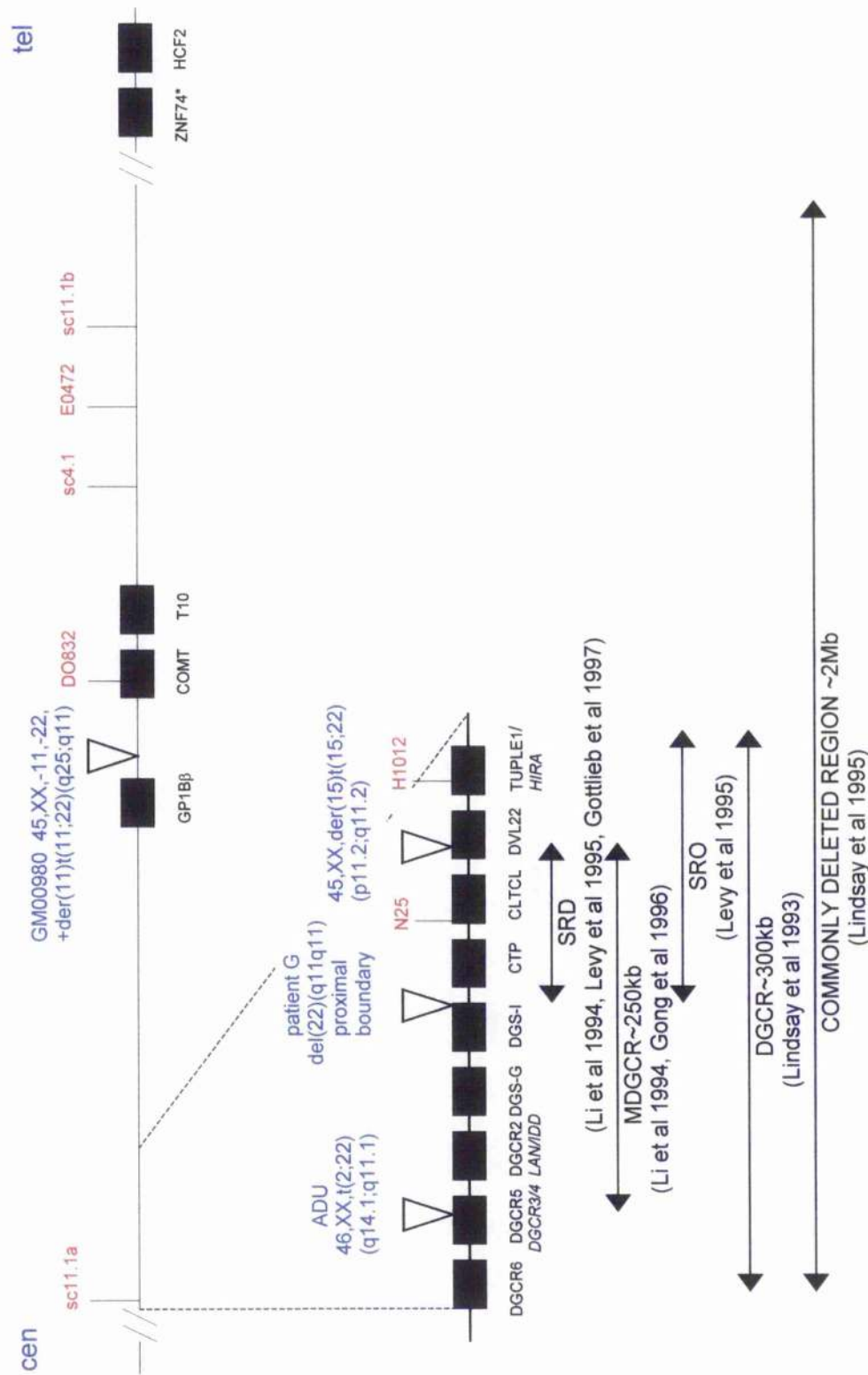


Fig. 1-11 Some of the markers and genes in the 22q11 deletion region. Adapted, with modifications, from Dallapiccola et al (1996), Gong et al (1996). *ZNF74 location according to Mulder et al (1995) and later references. Dallapiccola et al (1996) sites this more proximally, in the commonly deleted region. Some of the probes used to define deletions are marked in red. Breakpoints on abnormal chromosomes used to define the critical region are marked in blue.

Detection and Definition of DG/VCF Syndrome 22q11 Deletion

Even high resolution banding (at least 850 bands/haploid set) allowed cytogenetic identification of deletion in only 9/30 cases with hemizygosity at 22q11 (Wilson et al 1992b). More sensitive, molecular, detection initially employed RFLP analysis or quantitative Southern blotting. These techniques are time consuming and subject to variability and, by 1993, FISH was emerging as a particularly useful approach which allowed a more qualitative detection of deletion than Southern analysis and the relative ordering of markers (Lindsay et al 1993, Halford et al 1993a).

At the time of this study it was known that most patients with DG/VCF pathology had a large deletion detected by sc11.1, a probe which detects two loci (sc11.1a proximally and sc11.1b distally) approximately 2Mb apart (Halford et al 1993a, Lindsay et al 1993, 1995). Haplotype analysis of VCF patients had suggested deletions shared the same proximal breakpoint (between D22S427 proximally and D22S941) and one of two distal deletion breakpoints (between D22S944 and D22S264 or D22S311 and D22S306/308, Morrow et al 1995) but the precise extent of deletions was unknown. FISH analysis found one in forty four deleted VCF patients with deletion which included E0472 but not sc11.1b (Lindsay et al 1995).

Breakpoint analysis of unique smaller deletions allowed attempts to define a shortest region of overlap (Fig.1-11). The critical region was initially estimated as 300-575kb (Lindsay et al 1993, Halford et al 1993a). An unbalanced t(15;22) translocation then defined the minimal DiGeorge critical region (MDGCR, Li et al 1994, Gong et al 1996) as 250kb in the proximal part of the commonly deleted region which included the t(2;22) balanced translocation breakpoint found in DGS patient ADU, a prime candidate gene region (Lindsay et al 1993). However the atypical proximal deletion boundary in interstitially deleted DGS patient 'G' was positioned 100kb telomeric to this breakpoint, excluding it from the critical region (Levy et al 1995). If other factors are not the cause of DGS in this patient, the phenotypic effect of the ADU breakpoint could be due to its position effect on genes in the critical region (Levy et al 1995, Bedell et al 1996).

The identification of potentially causal genes was further complicated by the presence of another non-overlapping region, located distally in the commonly deleted region (Kurahashi et al 1996). Thus all the other transcription units in the commonly deleted region remained candidate causes. Among these, apparently lying just distal to the 250kb MDGCR (Gong et al 1996), is the *TUPLE 1* sequence (Halford et al 1993a) which, like N25, became a commonly used diagnostic FISH probe.

TUPLE 1 is also known as *HIRA*, the major splice variant of the same gene (Llevadot et al 1996). The *HIRA* protein product was implicated in transcriptional regulation based on its

similarity to other WD40-containing proteins which form multimeric complexes that function as transcription regulators, and because of its homology to Hir1p, Hir2p and Tup1p histone regulating transcriptional co-repressors in yeast (Halford et al 1993a, Lamour et al 1995). Homology to Hir1p and Hir2p indicated a possible role in histone synthesis regulation, and the fact that Tup1 bound to histones H3 and H4 suggested *HIRA* involvement in the regulation of chromatin structure and through this gene expression (Edmondson et al 1996). As *HIRA* protein was predicted to be a component of multi-protein complexes involved in transcriptional regulation, it was thought that its reduced level secondary to hemizyosity at 22q11 might alter the stoichiometric relationship with other subunits in the complex, which could conceivably disrupt function (Halford et al 1993a).

These features led to the proposal that haploinsufficiency for *TUPLE 1* was at least in part responsible for the DG/VCFS phenotype (Halford et al 1993a).

DG/VCF Syndrome Phenotype/Genotype Correlation

DG/VCFS is not a straightforward contiguous gene syndrome. The complexity of the phenotype does not correlate with deletion size (Scambler et al 1991). However, as almost all DG/VCFS deletions at 22q11 involve at least 250-300kb, non-overlapping regions can generate the phenotype, and, when this study began, no point mutations had been found in any gene in the commonly deleted region, it was suggested that it was not caused by a single transcription unit (Dallapiccola et al 1996).

As genes in the commonly deleted region have a similar expression pattern in embryogenesis it was further proposed that the entire region may behave as a functional unit so that the phenotype results from disruption of any of the genes or of some common regulatory element (Dallapiccola et al 1996). Position effects, such as the possible silencing effect of heterochromatin on proximally moved genes, might be involved. However, this concept of a functionally-related chromosome region did not preclude the existence of a major gene related to a specific phenotypic aspect.

1.7.1.1.2 Wolf-Hirschhorn Syndrome

Wolf-Hirschhorn syndrome (WHS, MIM 194190), which has an incidence of 1/50,000 live births, was first described in patients with cytogenetically visible deletion of 4p16-pter by Wolf et al and Hirschhorn et al (1965). Characteristic clinical features are severe growth retardation, severe mental retardation (MR), seizures, distinct facies (hypertelorism, highly arched eyebrows, sagging lower eyelids, short philtrum, carp-shaped mouth and most

notably a prominent glabella giving a characteristic Greek helmet profile), and a number of other anomalies including microcephaly, congenital cardiac defects, genital and renal abnormalities, simple ears, cleft lip and palate and ocular colobomata.

The chromosome 4p deletion arises as a *de novo* event in around 90% of cases, whereas in the remainder it is derived from a familial balanced translocation (Tranebjaerg et al 1984). At the start of this investigation, several WHS cases associated with a subtle or cryptic chromosome abnormality had been described (Altherr et al 1991, Gandelman et al 1992, Goodship et al 1992, Johnson et al 1994, Hagg et al 1992).

1.7.1.1.3 Rubinstein-Taybi Syndrome

Rubinstein-Taybi syndrome (RTS or RSTS, MIM 180849) is a multiple congenital anomaly syndrome characterised by mental and growth retardation, short broad thumbs and/or halluces, and typical facial features including downward-slanting palpebral fissures, abundant dark scalp hair and prominent columella (Michail et al 1957, Rubinstein and Taybi 1963). The existence of patients with an RTS phenotype and translocations involving chromosome region 16p13.3 identified disruption at this locus as one cause of the syndrome.

When this study began, a chromosome 16p13.3-specific cosmid sequence, RT1, had been found to be deleted in 25% (6/24) of RTS patients tested (Breuning et al 1993). The gene encoding the human CREB (cyclic AMP response element-binding) binding protein (CREBBP or CBP), located at 16p13.3, was subsequently cloned and it was shown that mutations in this gene are also associated with RTS (Petrij et al 1995).

1.7.1.2 Microdeletion Detection at the Dystrophin Locus in Duchenne and Becker Muscular Dystrophy Carriers

Duchenne muscular dystrophy (DMD) is an X-linked recessive disorder with a progressive, muscle-wasting course and fatal outcome before the age of 30 (reviewed in Emery 1993). Affecting 1 in 3500 live-born males, it is the most frequent neuromuscular disease, with one third of cases being due to new mutations. DMD is allelic with the milder and rarer Becker muscular dystrophy (BMD). The DMD/BMD gene encodes a membrane protein called dystrophin of approximately 400kDa (Hoffman et al 1987) and maps to chromosome band Xp21 (Francke et al 1985).

With 79 exons spanning 2.4Mb it is the largest recognised gene (Roberts et al 1993). In 60-70% of cases, DMD or BMD results from deletion (usually but not invariably cytogenetically cryptic) involving one or more exons. Duplications cause a further 6%. Breakpoints occurring in a major hotspot in the central part of the gene (intron 44-45) and a minor hotspot in the 5' part of the gene (around intron 7) account for 75% of all detected dystrophin gene deletions and duplications (Blonden et al 1991). Approximately 60% of deletions were believed to include exon 45, and occur in the region defined by the probe P20 (DXS269, Waapenaar et al 1988, Reid et al 1990).

Causal deletions are inherited in 60-70% of patients. Familial deletion is therefore implicated in ~40% of all cases, representing a high carrier risk for female relatives. Deletion detection by multiplex PCR is straightforward in affected males (Abbs et al 1991) but, when this present study commenced, the molecular approaches available for carrier detection in asymptomatic female relatives were limited and complicated by the presence of the normal X masking the result from the defective chromosome.

Identification of the chromosome segregating with the mutation by linkage analysis based on RFLPs required several intragenic and flanking probes due to the high frequency of intragenic recombination within the large dystrophin gene (12%). Highly polymorphic markers for linkage analysis were provided by PCR-amplified (CA)_n repeats (Clemens et al 1991, Bunyan et al 1994).

A definite carrier diagnosis could be achieved if size-altered 'junction' fragments (restriction fragments of altered mobility detected by cDNA probes on Southern blot) were demonstrable in an affected individual, but small rearrangements (e.g. one exon) could be missed (den Dunnen et al 1989). The use of PFGE or whole cosmid probes rather than short cDNA sequences (Blonden et al 1991) could increase the frequency of fragment identification. Junction fragments could also be detected with reverse transcription and nested PCR amplification (RT-PCR) of mRNA (Roberts et al 1990, Bunyan et al 1994). In cases with a defined deletion, investigators could employ gene dosage analysis in Southern blots (Darras and Franke 1988), in which the intensity of specific bands in test and control samples were compared by densitometry. However as band intensity is dependent on various experimental factors this carried a degree of uncertainty which many clinical laboratories considered unacceptable.

Demonstrating raised creatinine kinase (CK) levels could be diagnostic, but only 70% of adult carriers have raised CK so a normal level does not exclude carrier status (Sibert et al 1979). Immunostaining of muscle tissue by dystrophin antibodies was successful, but only for the detection of DMD, rather than BMD, carriers with elevated CK levels (Reid et al 1993).

In contrast to these often time-consuming, complex and potentially inconclusive approaches, and while a new approach, quantitative PCR, was in the early stages of introduction as a diagnostic tool (Mansfield et al 1993), a technically simple solution was offered by metaphase FISH with cosmid probes that are specific for commonly deleted regions of the dystrophin gene. This allowed direct and unequivocal determination of DMD/BMD carrier status in female relatives of known carriers or affected individuals, even when the CK or RFLP analysis was not informative (Reid et al 1990).

1.7.1.3 Detection of Subtelomeric Chromosomal Rearrangement

Apart from RTS and WHS, other genetic conditions including α thalassaemia with learning disability (ATR-16), cri du chat and Miller-Dieker syndromes have been associated with subtelomeric rearrangements (Lamb et al 1989, Overhauser et al 1989, Kuwano et al 1991). These rearrangements, including *de novo* subtelomeric deletions and unbalanced familial translocations, can be cytogenetically cryptic, and their detection initially required clinical suspicion to direct molecular or ISH testing of appropriate subtelomeric regions.

1.7.1.3.1 Screening for Cryptic Subtelomeric Rearrangements

As the recognised cause of much abnormality, gene-rich (Saccone et al 1992) and believed to be particularly prone to rearrangement, subtelomeric regions came to be considered appropriate loci for a focussed search for cryptic chromosomal rearrangements in the absence of a practical method of whole genome screening. These regions are G-band negative therefore rearrangements can be missed by conventional cytogenetics. Also the majority of chromosome translocations involve ends of chromosomes and subtelomere screening would detect all of these, regardless of size. When this study commenced, molecular methods, examining inheritance of VNTRs using Southern blotting, had allowed estimation that 6% of all cases of idiopathic MR were due to subtelomeric microdeletion (Flint et al 1995). This promised considerable diagnostic impact as MR, which affects approximately 3% of the population, is of unknown aetiology in around 40% of moderately to severely affected individuals (IQ<50) and 70% of people with mild learning disability (IQ 50-75) (Flint and Wilkie 1996).

A molecular screening test based on CA repeats would have facilitated such investigation, but primer development initially proved problematic. This, and the fact that FISH would

identify balanced rearrangements in carriers, led researchers to commence development of a FISH-based approach.

1.8 Comparative Genomic Hybridisation

Molecular genetic methods in the early 1990s were generally highly focussed, targeting one or at most a small group of chromosome region(s) at a time and leaving the majority of the genome unexamined. Studies were additionally restricted by, for example, polymorphic probe availability and informativeness, design of compatible sets of primers and differentiation of their product fragments. Conventional karyotyping does provide global (if low-resolution) analysis of the genome, but solid tumour analysis, in particular, is impeded by low quantity and quality of metaphase spreads, the complexity of genetic changes and the fact that some clinically significant chromosome abnormalities in cancers are not present in dividing cells. Identification of cytogenetic aberrations by FISH again needs to be guided by suspicion of the nature of the abnormality, depends on the availability of appropriate probes, and may require sequential hybridisations.

To circumvent these problems and, following some successful reverse painting with interspecies hybrid DNA (Boyle et al 1990, Kievits et al 1990), total genomic tumour DNA was used as a probe for CISS hybridisation to normal metaphases, identifying highly amplified sequences in some tumours (Joos et al 1993). Comparative genomic hybridisation (CGH), the first molecular cytogenetic technique to allow comprehensive analysis of the entire genome (Kallioniemi et al 1992b, du Manoir et al 1993), arose as a refinement of this approach (Fig. 1-12).

CGH is a 'one-step' method for quantitative genetic change determination and involves use of differentially labelled test and normal control DNA simultaneously as paints in CISS hybridisation to normal metaphase spreads. The test DNA (usually labelled or detected with a green fluor) and control DNA (labelled or detected with a red or orange fluor) hybridise in a concentration-dependent manner to chromosomal target sequences. Software integrates fluorescence from each label and calculates the ratio of green to red along each chromosome thereby identifying regions of DNA gain or loss. Ratio profiles can then be produced for each chromosome. The co-hybridisation of normal reference DNA improves sensitivity by providing a control for local variations in hybridisation efficiency possibly caused by differential denaturation of regions with different base composition and packaging or differing target accessibility (Bentz et al 1994).

Advantages of Comparative Genomic Hybridisation

As CGH is a global analysis which uses isolated genomic DNA the need for metaphase spreads is removed, obviating tissue culture which is time-consuming, may result in clonal loss and is not advisable with high-risk specimens. Abnormalities which are not present in PHA stimulated cells may be detected, and archived tissue studied (Speicher et al 1993). CGH can also assist interpretation in cases where chromosome preparations are available, but karyotypes are highly complex and difficult to analyse.

At the time of its introduction CGH was also faster than the molecular genetic approaches used to screen for genetic imbalance (e.g. loss of heterozygosity studies, quantitative Southern blot for amplification).

Critical Stages in CGH Procedure

i) Preparation of target metaphase chromosome spreads

The properties of the target metaphase preparations dramatically influence CGH results, possibly because each element of the probe is present at very low concentration so that accessibility of the target to the probe is critical (Kallioniemi et al 1994). Normal metaphase spreads for CGH targets are prepared from synchronised peripheral blood cultures from karyotypically normal donors (Wheater and Roberts 1987). On phase contrast microscopy, chromosome preparations should have a fairly high mitotic index with low cell density, little residual cytoplasm (which can cause high background and prevent optimal denaturation), be of adequate length (400-550 bands) and with minimal overlap (as CGH analysis excludes overlapped chromosomes). Longer chromosomes increase resolution but are more likely to intersect. Chromosomes should not have separated chromatids and, for good banding, should appear dark and not refringent. However, even preparations with apparently acceptable morphology may not produce acceptable CGH results. Researchers therefore may prepare several large batches of slides at once, ascertaining their suitability with CGH of two control DNAs. Suitable batches yield uniform, intense, painting of all euchromatic chromosome regions.

ii) Isolation of DNA

CGH is applicable to DNA from clinical samples such as blood, bone marrow or tissue, from cultured cells, or from histological sections. DOP-PCR can be used to amplify DNA from tissue sections (Speicher et al 1993) although the DNA fragments obtained may be of sub-optimal length for CGH.

iii) Probe labelling

Nick translation is the most widely used method of labelling DNA for CGH, allowing simple adjustment of fragment size by variation of reaction time or DNase:Polymerase I ratio. Originally, labelling was indirect, involving biotin and DIG-conjugated dUTPs visualised fluorescently via immunocytochemistry (Kallioniemi et al 1992b, 1994). Direct labelling subsequently expedited the process and minimised locus-specific hybridisation problems at 1p32-pter, 16p, 19 and 22 caused by differential ISH properties of DIG and biotin-labelled probes (Kallioniemi et al 1994, Bryndorf et al 1995).

The optimal genomic probe fragment length after nick translation (a smear from 600 to around 2000bp in an agarose gel) is longer than that for conventional FISH probes. This improves hybridisation intensity and uniformity.

iv) Hybridisation

Denaturation, incubation, post-ISH wash and slide mounting protocols essentially follow those used in basic FISH (Kallioniemi et al 1994). Target metaphases are denatured in 70% formamide at 72-74°C for ~3 minutes, though optimal time and temperature may vary between slide batches. The aim is maximal denaturation and penetrance while retaining chromosome structural integrity and good DAPI banding potential. Proteinase K slide treatment has been used to improve probe penetrance (du Manoir et al 1993, Kallioniemi et al 1994) but over-digestion may damage chromosome structure therefore proteolysis was increasingly omitted (as in the 1996 CGH procedure recommended by Vysis Inc, Downer's Grove, IL, USA).

Probes are generally equal mixtures of test and control DNA. The amount of each in 10ul hybridisation mix has ranged from 60ng (Kallioniemi et al 1992b) to 1ug (du Manoir et al 1993). Between 0.5 and 30ug/ul Cot1 DNA has been used in CISS CGH to block binding of labelled repetitive sequences to target metaphases. Hybridisation is carried out under a sealed coverslip in a moist chamber at 37-39°C for two to four days.

v) Fluorescence microscopy and image acquisition

The technical requirements for CGH three colour fluorescence microscopy are similar to those described for FISH analysis. High copy gene amplifications (of more than ten to twenty fold, Kallioniemi et al 1994) as well as changes involving large regions (e.g. entire chromosome arms) are visually detectable in homogeneous cell lines. More detailed evaluation is only possible using digital imaging.

Image acquisition follows selection of each of five to ten highest quality metaphases from each CGH area. Cooled monochrome CCD cameras offer high sensitivity, spatial

resolution and dynamic range with low noise, but cheaper video-rated cameras can provide adequate images for CGH analysis (du Manoir et al 1995).

CGH quality depends on illumination uniformity and accuracy of overlay of CCD images obtained with different filters. Signal visualisation was initially via a double bandpass (FITC/rhodamine) filter set or single bandpass filters aligned to minimize image shifts (Kallioniemi et al 1992b, du Manoir et al 1993). Fortunately, shortly after the introduction of CGH, the technology became available to optimise image registration by placing the excitation filter for each fluor in a computer-controlled filter wheel and viewing emitted light via a stationary triple bandpass filter. Grey-level images are obtained as Texas red/rhodamine, FITC and DAPI are sequentially excited, and exposure times are adjusted to obtain high intensity without saturation (Kallioniemi et al 1994).

Prior to analysis the image quality of captured metaphases is critically evaluated (Kallioniemi et al 1996). They should show uniform, smooth, intense, balanced green and red fluorescence. Background fluorescence around chromosomes should be low and uniform, and both chromatids of each chromosome and both chromosome homologues in each cell should show the same changes. Hybridisation to centromeres and heterochromatic regions must be minimal and chromosomes should show good software enhanced reverse DAPI bands. Images with nonspecific fluorescent spots on chromosomes are excluded from analysis, as are any showing inadequate denaturation as signified by fluorescence intensity variations which follow the DAPI banding pattern.

vi) Quantitative analysis

Quantitative analysis of green to red fluorescence ratios along chromosomes (described in Kallioniemi et al 1996) is performed using dedicated software, versions of which have been available since the mid 1990s from a number of commercial sources, e.g. Applied Imaging (Newcastle-upon-Tyne, UK) or Vysis.

Chromosomes are first segmented by adaptive thresholding (du Manoir et al 1993), providing a chromosome outline from which the medial axis is determined. Fluorescence intensities are then measured for the green and red images at one pixel intervals along the axes of individual chromosomes, each value representing the mean of the values from a strip perpendicular to the axis. Background fluorescence intensity surrounding the individual segmented chromosomes is determined and subtracted from the chromosome fluorescence. Fluorescence ratios are normalised so that the average green:red ratio for the entire metaphase spread is 1.0. The ratio of the fluorescence intensities (green to red) on individual chromosomes is calculated and chromosomal ratio profiles generated. With some, particularly earlier, software, the ratio values can be transformed into a three-colour lookup table, where different colours are assigned to deleted, amplified or

balanced chromosomal sequences depending on chosen thresholds (du Manoir et al 1993). This approach has been termed 'global' analysis (Digital Scientific, Cambridge, UK). In all systems, chromosomes are identified, for imbalance localisation, by software enhancement of the DAPI bands. Regions that show an increased green:red ratio have elevated copy number in the test DNA whereas those with a decreased ratio are deleted.

vii) Profile averaging

Slight differences in hybridisation quality between metaphase spreads and the presence of nonspecific signal limits the sensitivity of CGH analysis. It was recognised from the earliest use of CGH (Kallioniemi et al 1992b) that interpretation should be based on average ratio profiles for all homologous chromosomes from several cells in order to reliably detect smaller imbalances, but basic software for this task was not reported for another two years (Kallioniemi et al 1994). Initially, commercial CGH systems performed only rudimentary quantitative functions (e.g. Smartcapture, Digital Scientific) but it was apparent that automation of CGH processing would facilitate objective interpretation of CGH and its routine application, and systems evolved to perform most functions necessary for full CGH analysis. This includes interactive karyotyping and generation of a 'copy number karyotype' - a set of mean fluorescence profiles displayed to the side of the relevant chromosomal ideogram. At least four homologous chromosome profiles are included, after length normalisation, in an average profile (Kallioniemi et al 1994).

viii) Interpretation of CGH experiments

CGH result interpretation is based on whether the mean ratio value for a chromosomal region is outside a threshold (fixed or statistical). A fluorescence ratio of 0.5 corresponds theoretically to monosomy and 1.5 to trisomy, however in practice fixed thresholds are generally set between 1 ± 0.1 and 1 ± 0.25 . A range wider than 0.75-1.25 may be too insensitive to identify imbalances that are present in only a proportion of cells. A narrow range (e.g. 1 ± 0.1) might produce too many false positives. The first use of statistical thresholding involved tabulated comparison of average ratio values for each chromosome type against the limits of the 95% confidence interval (CI) calculated for the same chromosome type in a normal versus normal control CGH (du Manoir et al 1993). Initially ratio profile interpretation was not automated (Kallioniemi et al 1994). Software which displays apparently under- or over-represented regions (according to selected thresholds) as green or red bars against a karyotype ideogram was later developed, although interpretation was not standardised.

Sensitivity of CGH

Amplifications can be detected when the product of the copy number and size of the amplicon is at least 2Mb (Joos et al 1993). Under ideal conditions, the theoretical deletion detection limit is also 2Mb (Piper et al 1995), however several factors impair CGH resolution, particularly chromosome length variability. Lengths are normalised for each chromosome type before average ratio profiles are calculated, which results in slightly distorted map positions of identical regions on chromosomes of different lengths. In practice, therefore, 10Mb deletions are detectable (Kallioniemi et al 1992b, 1994). Sensitivity does depend largely on the threshold criteria selected to separate balanced from unbalanced regions in the test genome, and is affected by the degree of any mosaicism. Though theoretical ratios for monosomy and trisomy are 0.5 and 1.5, if only 50% of cells carry imbalance the ratios are 0.75 and 1.25. If less than 50% contain the imbalance, the ratio profiles are unlikely to reach the gain or loss thresholds. A compromise has to be made between sensitivity and specificity, depending on the experimental aims and availability of alternative methods of result confirmation. To detect imbalances present in only a proportion of cells a more permissive threshold may be required, with subsequent exclusion of false positives by, e.g., use of FISH probes.

Limitations of CGH

In addition to limitations imposed by mosaicism, CGH only detects aberrations involving loss or gain of DNA regions, not balanced translocations or inversions. It provides no information on the structural changes involved in gains and losses and, as the standard approach detects DNA sequence copy number changes relative to the average copy number in the entire specimen, it can not show alterations in total genome ploidy. Some regions are excluded from CGH analysis or, as 1p32-pter, 16p, 19 and 22, evaluated with particular caution. The repetitive DNA at heterochromatic regions, centromeres, and the short arms of acrocentric chromosomes is, due to polymorphisms, suppressed by Cot1 DNA to different extents in different individuals, and fluorescence intensity naturally declines at telomeres (Kallioniemi et al 1994).

Quality Assurance and Validation

Controlling hybridisation quality is more important in CGH than in other ISH techniques, because the result is analysed quantitatively. CGH quality is dependent on all steps of the procedure, the most difficult variable to control being the hybridisability of the target chromosomes. Variation in hybridisability affects CGH results by producing false positive findings (such as on 1p, 19 and 22) or by decreasing the dynamic range of the ratio

profiles (the amplitude of gains and losses). Only high quality images of high quality hybridisations, rigorously selected, should therefore be evaluated (Kallioniemi et al 1994, 1996).

Visual assessment of the images should show concordance with ratio profiles and green to red ratio should be consistent between metaphases. An estimation of the variation (e.g. standard deviation, SD) of the fluorescence ratios from one metaphase to another provides a measure of the result consistency and reliability. Ratio changes that clearly exceed the background ratio variation in a negative control CGH can be interpreted as evidence of real copy number differences. In a successful CGH the normal variation (± 1 SD) should not exceed ratios of 0.85 to 1.15 (Kallioniemi et al 1994, 1996).

Since most imbalances affect only a minority of the chromosomes, the normal range of ratio values can be deduced empirically from a number of chromosome types showing values close to that expected for balanced chromosomes (du Manoir et al 1993).

A positive control cell line with known imbalances of differing size can be used to evaluate the CGH sensitivity (Kallioniemi et al 1994).

Validation of CGH results can be achieved by re-examination of G-banding or by FISH (as in Bryndorf et al 1995) or conceivably, in the absence of cytogenetic preparations, by targeted molecular study. Repeat CGH, reversing the labels on test and control DNA, can also provide confirmation of true gain or loss (Kallioniemi et al 1994).

Application of CGH

CGH was primarily developed for and most widely applied in the study of solid tumours, providing characterisation of unbalanced rearrangements, implication of novel genes in cancer development or progression, analysis of clonal evolution and subclassification and prognostic evaluation of cancer (later reviewed in Forozan et al 1997). The technique was subsequently shown to be a powerful adjunct to conventional cytogenetics and FISH for characterisation of constitutional karyotypes with numerical aberrations or complex structural rearrangements unresolvable by G-banding (Bryndorf et al 1995). This group took six months to successfully establish CGH in their laboratory. Production of target metaphases of adequate quality for CGH was a particular problem, and they recognised that further technical development of CGH would be necessary for its routine clinical application. The present study concerned the introduction and application of CGH to the diagnostic service in this laboratory.

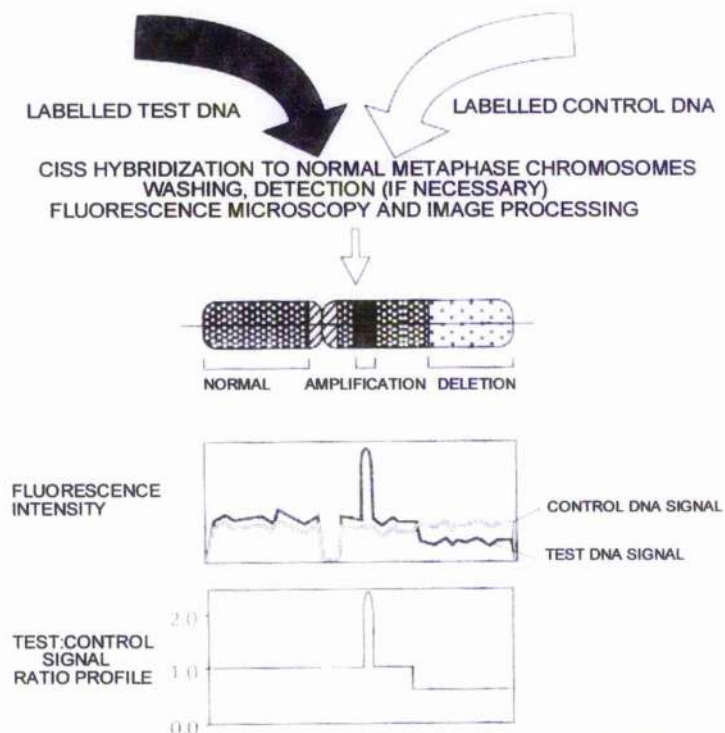


Fig.1-12 A schematic representation of CGH process and analysis. Adapted from Buckle and Kearney (1994).

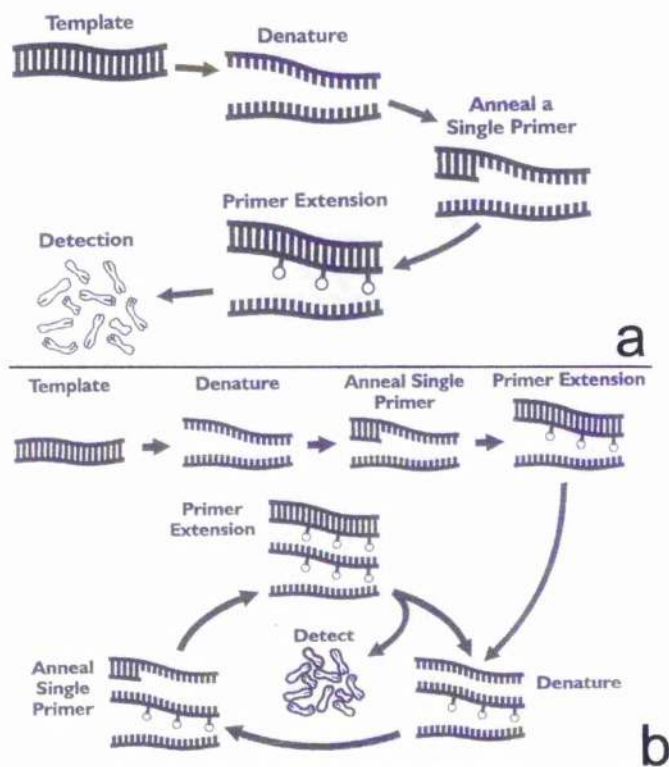


Fig.1-13 Diagram of PRINS (a) and Cycling PRINS (b). Primers are extended from their 3' ends. From A Guide to *In Situ* (1994), Hybaid Limited, Teddington, Middlesex.

1.9 Primed *In Situ* Labelling

PRimed IN Situ labelling (PRINS, Koch et al 1989, Gosden et al 1991) combines features of *in situ* hybridisation and the polymerase chain reaction.

1.9.1 The Polymerase Chain Reaction

The polymerase chain reaction (PCR) is a process for copying a specific DNA or cDNA target sequence (Saiki et al 1985, 1988, reviewed by Innis et al 1990). PCR is so sensitive that a single DNA molecule has been amplified, and single-copy genes are routinely derived from total genomic DNA then visualised as distinct bands on electrophoresis.

During the PCR the target DNA sequence is cycled through a series of incubation steps at different temperatures in a buffered solution containing thermostable DNA polymerase, $MgCl_2$, dNTPs and primers (a pair of oligonucleotides complementary to the 3' boundaries of the target sequence). This can be performed manually in preset water baths or automatically with a thermal cycler. In the first step, the double-stranded DNA target is heat denatured. In the second, the temperature is lowered to allow hybridisation of the primers to the single-stranded DNA and initiation of polymerisation. Raising the temperature slightly in the third step allows the enzyme to complete the copy initiated during this annealing. The annealing and extension steps can often be combined, giving a two-temperature cycle. Primers hybridise to opposite strands of the target sequence and are orientated so that DNA synthesis proceeds across the region between them, copying both target strands with each cycle. The thermal stability of the polymerase allows repetition of the cycle, with exponential accumulation of target DNA, as the copies subsequently act as templates.

PCR Reaction Mix

The most important chemical variable to optimise in a PCR is the free magnesium ion concentration in the reaction buffer. High concentrations enhance mispriming, causing production of nonspecific amplicons. At very low concentrations extension is impaired and yield reduced as Mg ions are a co-factor for DNA polymerase activity. The optimum Mg ion concentration must be determined by titration but is generally 1.5-3.5mM. Nucleotide concentrations of 20-200uM produce the optimal balance of yield, specificity and fidelity in solution PCR, and use of equivalent concentrations of the four dNTPs minimises misincorporation errors.

Up to 50mM KCl can be included in the reaction to facilitate primer annealing, but KCl above 50mM or NaCl at 50mM (as in stop buffer) inhibits the polymerase.

Inclusion of gelatin or bovine serum albumin (BSA) and nonionic detergents such as Tween 20 (0.05-1%) may improve enzyme thermostability.

PCR Enzymes

Thermus aquaticus (Taq) DNA polymerase, the most widely used PCR enzyme, has, at its optimum thermal range of 70-80°C, an extension rate of 35-100 nucleotides per second depending on the buffer, pH, salt concentration and nature of the DNA template. It has a 5'-3' exonuclease, which removes nucleotides ahead of the growing chain, but no proofreading 3'-5' exonuclease so its fidelity is low (2×10^{-4} errors per bp per duplication). Its optimal concentration is about 2 units (2U) per 100ul reaction. Use of over 4U can result in accumulation of nonspecific amplification products, whereas amounts less than 1U usually reduce specific product yield.

AmpliTaq (Roche Molecular Systems Inc, Branchburg, New Jersey, USA), encoded by a modified form of the Taq DNA polymerase gene, is recombinant, therefore the purity and reproducibility of this thermostable 94kDa enzyme is higher than Taq polymerase.

Dynazyme DNA polymerases (Finnzymes, Espoo, Finland), from *Thermus brockianus*, are less expensive than AmpliTaq and have increased thermal stability (150 minute half life at 96°C compared with Taq half life of 40 minutes at 95°C).

PCR Primers

Although selection of efficient, specific primers is fairly empiric, there are guidelines for their design and computer software, such as Primer 3 (frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi), to facilitate it. Primers should be 18-28 nucleotides long with an average G+C content of around 50% and random base distribution. Sequences with significant secondary structure and stretches of polypurines, polypyrimidines or other unusual sequences, such as palindromic sequences, should be avoided. Three or more Cs or Gs at 3' ends may promote mispriming at G+C-rich sequences, and complementarity at the 3' ends of primer pairs promotes formation of artefacts termed primer dimers and reduces yield of specific product.

Melting temperatures (T_m s) for a given primer pair should lie between 55°C and 80°C (calculated using 2°C for A or T and 4°C for G or C) and be balanced.

Primer concentrations between 0.1 and 0.5 μ M are generally suitable. Higher concentrations may promote mispriming and accumulation of nonspecific product and increase the probability of primer dimer generation. Nonspecific products and primer

dimers are also PCR substrates and compete with the target sequence for reactants, lowering specific product yield.

PCR Denaturation Time and Temperature

The temperature is usually monitored inside one reaction tube with a thermocouple probe. Typical denaturation conditions are 95°C for 30 seconds or 97°C for 15 seconds, however higher temperatures may be appropriate, especially for G+C-rich targets. The most likely cause of PCR failure is incomplete denaturation of the target template and/or PCR product. This allows DNA strands to reanneal, reducing yield. Preceding the first cycle with an initial 3 minute denaturation at 93°C may be beneficial, however denaturation steps that are too high or long reduce enzyme activity. Taq polymerase half life is >120, 40 and 5 minutes at 92.5°C, 95°C and 97.5°C respectively.

Primer Annealing

The temperature and time required for primer annealing depends on primer length, base composition and concentration. An applicable annealing temperature is 5°C below the primer T_m , with temperatures in the 55-72°C range generally giving optimal results. At typical primer concentrations (0.2μM), annealing requires only a few seconds.

As Taq polymerase is active over a wide temperature range, primer extension will occur at lower temperatures, including the annealing step. Increasing annealing temperature aids discrimination against incorrectly annealed primers and reduces misextension of incorrect nucleotides at primer 3' ends thereby promoting specificity. Specificity can also be improved in the initial cycle by adding the enzyme to the reaction mix at a temperature above that of primer annealing.

Primer Extension

Extension time depends on the length of the target being amplified. One minute at 72°C is sufficient for products of up to 2kb. Low extension temperatures and high dNTP concentrations favour primer misextension and extension of misincorporated dNTPs. The extension step can be omitted if the target is 150bp or less. During the thermal transition from annealing to denaturation, the sample will be in the 70-75°C range for the few seconds required to completely extend the annealed primers.

Cycle Number

When other parameters are optimised the optimum number of cycles primarily depends on the starting concentration of target DNA. Too many can increase the amount of nonspecific products (see below) and too few reduce product yield.

Plateau Effect

This is the attenuation in the exponential rate of product accumulation that occurs during late PCR cycles with the build-up of 0.3-1pmol of specific product. Depending on reaction conditions, the factors influencing the 'plateau effect' include substrate utilisation, dNTP or enzyme stability, end-product inhibition, competition for reactants by nonspecific products or primer dimer, reannealing of specific product at concentrations over 10^{-8} M and incomplete denaturation/strand separation of product at high product concentration. An outcome of the plateau, averted by optimising cycle number, is that an initially low concentration of nonspecific products may continue to amplify preferentially.

Contamination in PCR

As PCR produces highly amplified product from a small amount of DNA it is particularly affected by DNA contamination. Experiments should therefore be set up in a laminar flow cabinet or separate laboratory and dedicated PCR equipment and reagents used. Disposable gloves and lab coat must be worn. Where appropriate, reagents should be sterilised in an autoclave. Reaction tubes and reactants (excluding sample DNA, primer and enzyme), should be irradiated with UV light (Newton and Graham 1994).

1.9.2 The PRINS Procedure

Amplification *in situ* in non-disrupted cells allowing subsequent detection of the amplicon at the site of origin has been proposed since the earliest descriptions of PCR. In PRINS, sequence-specific primers are annealed to DNA in slide-bound chromosomes or nuclei then extension, incorporating labelled dNTPs, is catalysed by thermostable polymerase using chromosomal DNA as a template (Koch et al 1989, Fig.1-13).

Both oligonucleotide and double stranded DNA fragments have been used as primers in PRINS. Synthesised oligonucleotides (used singly or, if this improves specificity, paired, Gosden and Lawson 1994) are generally 15-30bp (Gosden et al 1991). Double stranded PRINS primers are usually a few hundred base pairs long (Koch et al 1991).

PRINS with alpha satellite-specific primers was made more convenient with the introduction, in 1995-96, of commercial oligonucleotides and labelling kits (Boehringer-Mannheim (Roche), Advanced Biotechnologies).

Sample Preparation for PRINS

Sample preparation must protect against loss of target morphology during the extreme temperatures used in PRINS. Alcohol and acid/alcohol fixatives, and hence standard cytogenetic slide preparations, can be utilised. Slides are generally used within one week of preparation. Prior to PRINS, dehydration of such fresh preparations in an alcohol series is crucial for maintenance of sample morphology.

PRINS Reaction Mix

As with solution phase PCR, the optimal reaction mix composition must be empirically determined for each PRINS target. Higher concentrations of $MgCl_2$ (up to 4.5mM), primer or enzyme may be needed for PRINS than solution PCR (according to Roche/Perkin Elmer).

A typical reaction mix contains 100-200uM dATP, dCTP and dGTP and 100-250uM labelled dUTP (as in Gosden and Hanratty 1993, Koch 1996). Biotin, DIG, or FITC label can be employed. Use of FITC-dUTP may produce weaker signal than hapten-labelled nucleotides, but associated background signal reduction can maintain signal to noise ratio (Koch et al 1992).

Solution PCR primers can be successfully employed in PRINS and generally 100ng-1ug oligonucleotide or 1-3ug of cloned probe is used per 50ul reaction mix (Koch 1996). One unit of DNA polymerase per 50ul reaction is usually adequate. Reaction buffer which provides optimal pH and ionic strength for PCR is often supplied with the enzyme. Inclusion of BSA or gelatin may enhance PCR efficiency, probably by blocking nonspecific binding sites in the sample or on the slide that could sequester reactants, but such carrier protein can denature and interfere with amplification.

AmpliTaq DNA Polymerase /S

Successful PRINS often requires a higher concentration of enzyme than solution PCR. AmpliTaq DNA Polymerase /S (Roche), a component of the GeneAmp Kit (Perkin Elmer, Foster City, CA, USA) is supplied at 20U/ul in a low detergent buffer and is optimised specifically for PCR *in situ*. It can be used at up to 10U/50ul without introducing significant concentrations of detergent from the buffer into the reaction, minimising sample damage caused by detergent during prolonged exposure in cycling (see below).

PRINS Denaturation, Annealing and Extension Temperatures and Times

Slides may undergo preliminary denaturation in 70% formamide solution at 70°C for 2 minutes followed by 70% (-20°C), 90% and 100% (room temperature) ethanol and air drying (Gosden and Lawson 1994). Reaction mix is then applied and slides placed at the annealing temperature. Alternatively denaturation is performed on a thermal cycler block. The slides either have reaction mix applied prior to placing on the hot block (Advanced Biotechnologies Print Kits) or the slide is placed on the hot block to preheat to denaturation temperature before the mix is added and the reaction begins (Koch 1996). As in solution PCR, when reactants are added to the target DNA at temperatures below the optimal annealing temperature, mispriming and primer-dimerisation can occur, reducing specific signal and contributing to background. Denaturation is at 93-94°C for up to 5 minutes. Temperature is then lowered (to a level depending on the probe used and stringency required) to allow probe annealing. If a programmable hotplate is not used the slide must be rapidly transferred between hotplates to avoid temperature dropping below annealing temperature. Annealing is for 2-60 minutes (according to probe amount, size and complexity) but 15-30 minutes is often suitable. The temperature may be raised for a separate extension stage, if required. In single cycle PRINS, the reaction is stopped on completion of extension.

PRINS is terminated by immersion in stop buffer containing EDTA at or just above annealing temperature. Following washing, the slide is ready for mounting in counterstain and microscopy if a fluorescent nucleotide was incorporated. Detection with antibodies or (strept)avidin is necessary for hapten labels.

Detection of Labelled Products

When DIG- or biotin-labelled dUTP has been incorporated into the amplified target sequence the slides are treated with blocking buffer then the appropriate reporter applied (see DIG and biotin detection in Methods). Slides are then mounted in counterstain and visualised using fluorescence microscopy.

Optimisation and Control of PRINS Reactions (Koch 1996, Hybaid Workshop, Edinburgh, 1996)

To assure PRINS specificity and sensitivity it is essential to stringently maintain reaction temperature on the slide during denaturation, annealing/extension and incubation in stop buffer, and a programmable slide thermal cycler improves result reproducibility.

Optimisation of the denaturation and labelling temperature may be required, adjusting temperatures only by small increments in each test. Absent or weak signals may be

caused by a denaturation temperature which is too low. This can be elevated, to a maximum of 95°C, or the duration of the step increased, to a maximum of 5 minutes, however excessive denaturation will destroy chromosome morphology. Overly high temperature during annealing/extension leads to inefficient priming, also resulting in poor signal, and even brief exposure of denatured target DNA to temperatures below the stringent annealing/extension temperature before and during extension and between extension and stop buffer can cause nonspecific priming and background signals. In addition to these temperature effects, DNA repair, mispriming and detection system artefacts can contribute to false positive results and failure of reactants can give false negatives. Thus appropriate controls should be employed, such as inclusion of known positive and negative samples, especially for interphase analysis or for metaphase studies (e.g. of sex chromosomes) where there is no internal positive control. Appropriate control investigations may identify the cause of sub-optimal results. Where there is nonspecific signal only on chromosomes, primer omission will reveal any 'endogenous priming' (label incorporation in damaged or nicked DNA via nick translation *in situ* by the polymerase's DNA repair mechanism). If found, slides freshly prepared from well-fixed material should be used to minimise damage. Exposure of the preparation to nucleases during fixation and pretreatment can be reduced by using high-quality sterile reagents. Alternatively, nicks in chromosomal DNA can be inactivated by incubation with a termination solution (DNA polymerase and dideoxynucleotides) or closed with T4 DNA ligase prior to denaturation (as in Gosden and Lawson 1994). Where there is nonspecific signal throughout the slide, controls could include detection without prior PRINS, which demonstrates nonspecific binding of detection agents, and omitting enzyme and primer to show nonspecific binding of label. If either test is positive, blocking and washing can be intensified, but repeating the chromosome preparation may be required to remove the excess cellular proteins and matrix responsible. If specific signals are large but minor additional signals are visible, increasing the dTTP concentration can conveniently reduce reaction sensitivity. If specific signal is weak it may be enhanced by further layers of detection, but this may also increase background.

Advantages of PRINS

PRINS is rapid, detection of highly repetitive DNAs on chromosomes taking only 2 hours (Gosden and Lawson 1994). It is highly specific, so that a single nucleotide mismatch with the genomic sequence at the 3' primer end prevents amplification, potentially allowing discrimination between two target sequences that differ by only one base (Pellestor et al 1994). It is also very efficient, the small size of the primers maximising

sample penetration and target hybridisation (Pellestor et al 1995a), and the short PRINS incubation steps may preserve chromosome morphology (Koch et al 1992).

As they are unlabelled, excessive amounts of primers can be used in PRINS to optimise reaction kinetics without increasing background. Unincorporated labelled dNTPs do not produce visible signal and, in good quality preparations, should only be incorporated at the primer annealing site so rarely cause nonspecific fluorescence. Non-hybridised or poorly-hybridised FISH probes, however, can generate background.

The preparation of oligonucleotide primer using an automatic DNA synthesiser is fast and relatively inexpensive whereas FISH probe production is laborious and involves procedures which are not accessible for all labs. Also, FISH probes then require to be labelled before use, and commercial probes are expensive and the selection limited.

Limitations of PRINS

When first introduced, PRINS had limited application because it was only reliable for detection of moderate to high copy repeats e.g. alphoid sequences, as the product had to be greater than 1kb. Also, only one locus could be detected.

Modifications of PRINS

PRINS has undergone development to improve its sensitivity and versatility. The process was extended to low copy repeat sequences with 'cycling PRINS', also termed *in situ* PCR, where multiple amplification rounds are performed by denaturing the initial product from its target, annealing of more primer and further extension, generating more product (Gosden and Hanratty 1993). An example of such a reaction would be annealing at 60°C for 3 minutes, extension at 70°C for 10 minutes, then up to 30 cycles with denaturation at 95°C for 1 minute, annealing at 60°C for 1 minute, and extension at 70°C for 3 minutes. 'Instant' and 'flash' modifications allowed further acceleration of PRINS, producing visualisation of high copy repeats in 15-20 and 2-3 minutes respectively (Gosden and Lawson 1995). This was achieved by incorporating fluor-labelled dUTP in the product during synthesis, obviating hapten detection, and removing initial formamide denaturation, using only heat denaturation on a thermal cycler. The 'instant' program was then 95°C for 1 minute, 60°C for 1 minute, and 70°C for 5 minutes. 'Flash' PRINS had even shorter denaturation, annealing and extension (30, 30, and 60 seconds).

Multi-PRINS (detection of several chromosomes simultaneously) required multiple sequential primer reactions, each time using a different labelled nucleotide, blocking nicks and free 3' ends of previously synthesised DNA with dideoxynucleotides (ddNTPs) to prevent their use as initiation sites (Gosden and Lawson 1994, Speel et al 1995).

Applications of PRINS and Cycling PRINS

In addition to metaphase chromosomes and nuclei, PRINS and *in situ* PCR has been used on a variety of preparations including uncultured blood cells (Gosden and Scopes 1996), spermatozoa and blastomeres (Pellestor et al 1995a, 1996a,b), tissue sections (Speel et al 1996), cells in suspension and cytopins (Komminoth et al 1992).

An obvious application is mapping and investigation of repeat sequence organisation within chromosomes (Gosden et al 1991). Multi-PRINS involving Alu primers can provide simultaneous R-banding for such mapping (Gosden et al 1991, Speel et al 1995) and PRINS with consecutive chromosome painting can be performed as a single procedure (Hindkjaer et al 1995a).

Centromere and telomere-specific PRINS has been used to assist characterisation of acquired and constitutional structural chromosome abnormalities (Hindkjaer et al 1995b, Brandt et al 1993, Hertz et al 1995, Friedrich et al 1996), in supernumerary marker chromosome identification (Koch et al 1993), and in studying the frequency and distribution of aneuploidy in human sperm (Pellestor et al 1995a, 1996a).

PRINS could potentially provide the speed and accuracy required by PND (Pellestor et al 1995b,c) and PGD (Pellestor et al 1996b) of sex or aneuploidy. The design of primers capable of 13 and 21 alpha satellite discrimination appeared to enhance PRINS suitability for this purpose (Pellestor et al 1994, 1995b).

The ultimate aim of PRINS development has been the detection of unique sequences. This would offer a means of chromosomal localisation for sequences defined only by pairs of primers (e.g. STSs and ESTs), obviating inter-lab clone exchange and long-term storage with the accompanying risks of mutation or inactivation. Most significantly, PRINS specificity would provide detection of small deletions (outwith FISH resolution) or even mutations. By the start of this study unique sequence PRINS had been reported, but only in a limited number of publications (Cinti et al 1993, Troyer et al 1994a,b). Only one of these reports involved human chromosomes and fluorescence, rather than chromogenic, detection (Cinti et al 1993).

1.10 Aims of the Study

Fundamental to this work was the development and application in this department of reliable chromosomal localisation, by FISH, of unique DNA sequences in a range of different vectors. This expertise could then be directed to the institution of a departmental facility which could validate and utilise non-commercial FISH probes for chromosomal breakpoint investigation. It could also, in the absence of appropriate commercial probes, be applied in the establishment and provision of a diagnostic microdeletion detection service.

A particular clinical need was identified for microdeletion determination in patients with DG/VCFS, because of the high incidence of this disorder, and in potential carriers of DMD or BMD, because FISH could offer the most direct and unequivocal diagnosis in cases with recognised familial microdeletions. Probes for these conditions, in addition to RTS, were fortunately available from other research groups.

Detection of cryptic chromosomal abnormalities acquired heightened clinical relevance with the report that ~6% of cases of idiopathic MR were caused by subtelomeric segmental aneusomy (Flint et al 1995). This study therefore incorporated the introduction of a novel multiple subtelomeric probe system and appraisal of this approach in the investigation of patients with idiopathic MR.

Although CGH had been used as a research tool in this department, the work reported here was intended to optimise CGH for reliable and convenient investigation, in a routine diagnostic environment, of apparent or suspected unbalanced constitutional chromosome abnormalities.

The study also aimed to introduce PRINS to this laboratory, and, in particular, investigate its potential use for the detection of single copy sequences.

2. MATERIALS AND METHODS

2.1 Safety

Protective coat and gloves were worn during all laboratory work. Handling of all unfixed biological material was carried out in a Class II safety cabinet. Work with toxic and carcinogenic agents was performed with care in a fume hood according to departmental safety guidelines.

2.2 Samples

The cytogenetics laboratories at the Department of Medical Genetics, Yorkhill NHS Trust supplied fixed metaphase preparations remaining after diagnostic analysis and also the results of these analyses. The molecular genetics laboratory provided DNA for CGH studies. Patient sample details are provided in Results and Appendix II.

2.3 Commonly Used Solutions

TE Buffer (10mM Tris-HCl, pH8.0, 1mM EDTA pH8.0).
A commercial version (x100, Sigma T-9285) was also used.

Phosphate Buffered Saline (PBS)
Dissolve ten PBS tablets (Dulbecco) in 1L water. Adjust pH to 7.4 prior to sterilisation by autoclaving.

pH6.8 buffer
Dissolve one pH6.8 buffer tablet (Gurr, BDH) in 1L purified water.

20xSSC (3M Sodium chloride, 0.3M Trisodium citrate)
Dissolve 175.3g of Sodium chloride and 88.2g of Trisodium citrate in 800ml purified water. Adjust the pH to 7 with a few drops of 10M Sodium hydroxide. Adjust the volume to 1L with purified water. Sterilise by autoclaving. Store at room temperature.
A commercial 20xSSC solution (GibcoBRL 15557-036) was also used.

2xSSC
Add 50ml 20xSSC to 450ml purified water.

0.4xSSC
Add 10ml 2xSSC to 40ml purified water in a Coplin jar.

0.4xSSCT (0.4xSSC, 0.15% Tween 20)
Add 10ml 2xSSC to 40ml purified water, 75ul Tween 20 (Sigma P-1379) in a Coplin jar.

4XT (4xSSC, 0.05% Tween 20)

Add 250ul Tween 20 to 500ml 4xSSC (100ml 20xSSC, 400ml purified water).

70% formamide solution (toxic)

Add 35ml formamide (Fluka 47670) to 5ml 20xSSC and 10ml purified water in a Coplin jar.

50% formamide solution (toxic)

Add 25ml formamide (Fluka 47670) to 5ml 20xSSC and 20ml purified water in a Coplin jar.

DAPI (4',6-diamidino-2-phenylindole dihydrochloride)

Prepare 40ug/ml stock by diluting 1ul DAPI 1mg/ml (Sigma D-1388) in 24ul sterilised distilled water. Store, covered to protect from light, at 4°C.

DAPI counterstain

Dilute stock, e.g. 1/50 to 1/25 (0.4ul DAPI 40ug/ml, 9.6ul mountant), in Citifluor AF1 mountant (Citifluor Ltd). This gives a final concentration of 0.8-1.6ug/ml.

DAPI + Propidium iodide (PI, toxic) counterstain

1/50 dilution of 40ug/ml DAPI plus 1/50 dilution of 20ug/ml Propidium iodide (Sigma P-4170) in Citifluor AF1.

Carnoy's fixative (1:3 glacial acetic acid:methanol)

2.4 Probes

Probes for these studies were provided, together with associated information, by many different sources. These are detailed in Results and Appendix I.

2.5 Plasmid, Cosmid, BAC and PAC Probe Culture

Materials

LB (Luria-Bertani) Culture Medium

Bacto-tryptone (Difco 0123-17-3)	5.0g
Bacto-yeast extract (Difco 0127-17-9)	2.5g
NaCl	5.0g

Instead, for convenience and as Difco is no longer trading, LB tablets (Sigma L-7276, 1 tablet makes 50ml medium) were used for BAC and PAC clone culture.

Add purified water to 500ml, autoclave to sterilise. Cool and add appropriate antibiotic, either:

1ml of 50mg/ml ampicillin (Sigma A-9518), prepared by adding 1g to 20ml purified water and filter sterilisation (0.22µ Millex filter unit, Millipore, Cork, Ireland). Final concentration in medium 0.1mg/ml.

1ml of 7.5mg/ml kanamycin (Sigma K-4000). Final concentration 0.015 mg/ml.

500ul of 20mg/ml chloramphenicol (Sigma C-7795), prepared by adding 1ml filtered ethanol (0.45µ Millex filter unit) to 20mg in supplied vial. Final concentration 0.02mg/ml.

LB Agar Plate Preparation

Add 7.5g (1.5%) Bacto-agar (Difco) to 500ml LB medium. Alternatively add ten LB Agar tablets (Sigma L-7025) to 500ml purified water. Autoclave to sterilise.

Add antibiotic when bottle is just cool enough to hold (ampicillin to 0.1mg/ml, kanamycin to 0.015mg/ml, chloramphenicol to 0.02mg/ml). Makes about 20 x 100mm plates.

Procedure

1. Streak out stab culture on agar plates containing appropriate antibiotic. Incubate overnight at 37°C.
2. Prepare starter cultures by transferring individual colonies to 5ml selective LB medium in 30ml Universal containers (Sterilin). Incubate 8-16 hours, 37°C, shaking (~300rpm) in an orbital incubator (e.g. S150, Bibby, Stuart Scientific, UK).
3. Set up glycerol stocks (50:50 culture:sterile glycerol (Sigma G5516)) in 2ml freezing vials (Sarstedt) for storage at -20 to -70°C.

2.6 Plasmid, Cosmid, BAC and PAC Extraction and Isolation

Probe extraction and isolation initially used the Circleprep kit (almost all DMD probes were isolated by this method) but this product was discontinued, leading to trial of the Hybaid midiprep system. This was compared with Circleprep for extraction of cosmid cYD4.66. Probe signal was weaker, with more background. Four subclones of DG/VCFS probe H1012 were then used for comparison of Circleprep with the Qiagen midiprep kit. Though slightly more background occurred using Qiagen, the quality was acceptable and this kit adopted for subsequent probe extractions.

2.6.1 Isolation of Cosmid DNA with the Circleprep II Kit (Bio 101 Inc. CP200)

Materials (*stored at room temperature to avoid precipitation)

Isopropanol (Sigma I9516)

Kit contents:

Pre-lysis buffer (Tris/EDTA/glucose solution)

Alkaline lysis reagent (0.2N NaOH and 1% SDS)*

Neutralizing solution (3M potassium acetate)

Lithium chloride (LiCl) solution*

Circleprep Glassmilk

Binding buffer (KBr/NaI/Tris mix, promotes binding of DNA to Glassmilk)

Wash solution (30ml of salt solution, 30ml of 100% ethanol added before use)

RNase MiXX x10

Sieve material

Procedure (essentially according to manufacturer's protocol)

1. Inoculate 100mls selective LB broth in a sterile bottle (e.g. 280ml tissue culture flask) with 100ul starter culture or 200ul glycerol stock and grow 8-16 hours, shaking at 37°C.
2. Centrifuge (Sorvall RC-5B Refrigerated Superspeed Centrifuge, HB4 rotor) in closed 50ml tubes (Oakridge (Nalgene) Nalge Company, Rochester, New York) at 6000rpm for 15min to pellet cells. Discard supernatant, drain tube for 1-2min.
3. Resuspend pellet with 4ml pre-lysis buffer and vortex to mix.
4. Add 4ml alkaline lysis reagent, mix immediately and incubate at 45-65°C, 5min.
5. Add 4ml neutralizing solution. Invert by hand until white flakes appear homogeneous. Centrifuge, 12,000rpm (~20,000g) for 5min at 4°C to pellet precipitate.
6. Transfer supernatant through sieve material provided to a clean 50ml tube.
7. Add 12ml isopropanol. Mix. Centrifuge 12,000rpm, 10min, 4°C. Decant supernatant. Centrifuge. Remove last of supernatant with a micropipette. Invert tube and wipe inside walls with tissue. Resuspend pellet in 0.5ml water, transfer to a microfuge tube.
8. Puncture tube lid, place in hotblock at 100°C, boil 3min to denature linear cellular DNA without nicking vector.
9. Cool on ice 30 seconds.
10. Add 300ul LiCl. Mix and let stand, room temperature, 5min. Centrifuge in a microfuge at 13,000rpm, 30 seconds. Transfer supernatant to a clean tube.
11. Add 600ul isopropanol to supernatant, mix. Microfuge 1min, resuspend pellet in 0.5ml water. Add 20ul RNase MiXX, incubate 37°C, 5min.
12. Add 300ul LiCl and 75ul vortexed Circleprep Glassmilk. Incubate 5min at room temperature with occasional mixing. Microfuge 5 seconds to pellet Glassmilk. Remove supernatant.
13. Wash pellet twice with 1ml wash solution and remove the residue of this solution.

14. To elute plasmid from Glassmilk resuspend pellet in 100ul sterile purified water, incubate in a 45-65°C waterbath for 5min. Microfuge for 1min and transfer supernatant containing vector DNA to a clean tube.

2.6.2 Isolation of Cosmid DNA with the Qiagen Midiprep Kit

Materials

Supplied Buffers (all stored at room temperature except P1 with RNase A):

Buffer P1 (Resuspension Buffer, store at 2-8°C after addition of RNase A) - 50mM Tris-HCl pH8, 10mM EDTA, 100ug/ml RNase A

Buffer P2 (Lysis Buffer) - 200mM NaOH, 1% SDS

Buffer P3 (Neutralisation Buffer) - 3M Potassium acetate, pH5.5

Buffer QBT (Equilibration Buffer) - 750mM NaCl, 50mM MOPS pH 7.0, 15% isopropanol, 0.15% Triton X-100

Buffer QC (Wash Buffer) - 1.0M NaCl, 50mM MOPS pH 7.0, 15% isopropanol

Buffer QF (Elution Buffer) - 1.25M NaCl, 50mM MOPS, pH 7.0, 15% isopropanol

Procedure (essentially according to manufacturer's protocol)

Before starting:

- Add RNase A vial (briefly centrifuged) to the P1. Gives a concentration of 100ug/ml.
 - Check P2 for SDS precipitation. If necessary, dissolve the SDS by warming to 37°C.
 - Pre-chill P3 to 4°C.
1. Follow Circleprep Method steps 1 and 2.
 2. Completely resuspend the bacterial pellet by vortexing in 4ml P1.
 3. Add 4ml P2, mix gently but thoroughly by inverting 4-6 times. Incubate at room temperature for 5min, no longer. The lysate should appear viscous.
 4. Add 4ml chilled P3, gently mix immediately by inverting 4-6 times, and incubate on ice for 15min. A white precipitate, containing genomic DNA, proteins, cell debris and SDS, forms and the lysate becomes less viscous.
 5. Centrifuge, immediately after mixing again, in polypropylene tubes at 12,000rpm in a Sorvall RC-5B HB4 rotor (~20,000g) for 30min at 4°C. Remove supernatant, which should be clear, promptly.
 6. Re-centrifuge supernatant at 12,000rpm, 15min, 4°C. Remove supernatant promptly.

7. Equilibrate a Qiagen-tip 100 by applying 4ml QBT, and allow column to drain.
8. Without delay apply the supernatant from step 6 to the Qiagen-tip.
9. Wash the Qiagen-tip with 2x10ml QC.
10. Elute DNA with 5ml QF. Collect eluate in an Oakridge tube (polycarbonate tubes are not resistant to alcohol). This may be stored overnight at 4°C.
11. Precipitate DNA by adding 3.5ml room-temperature isopropanol to the eluted DNA. Mix, centrifuge immediately at 12,000rpm for 30min at 4°C. Carefully decant supernatant.
12. Wash pellet with 2ml 70% ethanol, and centrifuge at 12,000rpm for 10min. Carefully decant the supernatant.
13. Air dry the pellet for 5-10 min and re-dissolve the DNA in 100ul TE buffer, pH8.0.

2.6.3 DNA Isolation From BAC and PAC Clones

Adapted from Children's Hospital Oakland Research Institute method, a modification of the standard Qiagen procedure that uses no organic extractions or columns (<http://bacpac.chori.org/bacpacmini.htm>).

Solutions

P1, P2, P3 as in Qiagen Midiprep Kit

Method

1. Using a disposable loop, inoculate a single bacterial colony into 5ml LB media plus appropriate antibiotic. Incubate at 37°C for 8-16 hours, shaking at 225-300rpm.
2. Prepare glycerol stock as described above (section 2.5) then transfer 2ml of culture to a 2ml Apex tube (Alpha Laboratories). Microfuge this 2ml aliquot at 13,000rpm for 3min.
3. Discard supernatants. Drain tube. Resuspend (vortex) pellet in 0.3ml P1. Add 0.3ml P2 and gently shake to mix. Let sit at room temperature for at least 5min. The appearance of the suspension should change from very turbid to almost translucent.
4. Slowly add 0.3ml P3 and gently shake during addition. A thick white precipitate of protein and E. coli DNA forms. Place the tubes on ice for at least 5min.
5. Microfuge at 13,000rpm for 10min at 4°C.
6. Remove from centrifuge and place on ice. Transfer supernatant, avoiding white precipitate, to a 1.5ml microfuge tube using a micropipette. Add 0.8ml ice-cold isopropanol. Mix by inverting tube a few times and place on ice for at least 5min. Can be left at -20°C overnight.
7. Microfuge at 13,000rpm for 15min at 4°C.

8. Remove supernatant and add 0.5ml of 70% ethanol. Invert several times to wash the pellet. Microfuge 13,000rpm for 5min at 4°C.
9. Repeat step 8. Remove as much of the supernatant as possible.
10. Air dry pellet at room temperature. When pellet becomes translucent, i.e. when most of the ethanol has evaporated, add 40ul TE. Occasionally tap the bottom of the tube to gently resuspend. Resuspension may take over 1 hour.

2.7 Calculation of DNA Concentration

1 unit of absorbance at 260nm = 50ug double stranded DNA/ml.

The spectrophotometer (Gene Quant II RNA/DNA Calculator, Pharmacia Biotech) was set to read absorbance at 260nm. A 1ml, 1/200 dilution of DNA in ultrapure water was prepared. A blank of ultrapure water in a quartz cuvette was used to set the background absorbance to zero. Absorbance of the test DNA in a matched cuvette was then read and concentration (ug/ml) calculated as absorbance at 260nm x 50 x dilution factor (200). For example: $0.034 \times 50 \times 200 = 340\text{ug/ml} = 0.34\text{ug/ul}$.

2.8 Probe Labelling

Nick Translation with Gibco BRL Kit (Catalogue number 8160-010)

Procedure (essentially according to manufacturer's protocol)

1. Add sequentially to a microfuge tube:
 - 5ul kit solution A4 (0.2mM nucleotides C, G and A in 500mM Tris-HCl (pH7.8), 50mM Magnesium chloride, 100 mM 2-mercaptoethanol, 100ug/ml bovine serum albumin
 - 1ug DNA to be labelled
 - 3.5ul 0.3mM Biotin-11-dUTP (Sigma B6780)
 - Solution E (water) to make final volume 50ul
 - 5ul solution C (0.5U/ul DNA Polymerase I, 0.4mU/ul DNase I, 50 mM Tris-HCl (pH 7.5), 5mM Magnesium acetate, 0.1mM phenylmethylsulphonyl fluoride, 50% glycerol, 100ug/ml bovine serum albumin
2. Mix well then incubate for 90min at 15°C.
3. To stop the reaction add 5ul solution D (300mM diSodium EDTA, pH8).
4. To precipitate the probe DNA add:
 - 4.6ul 3M Sodium acetate pH5.2 (filter sterilised, Sigma S-7899)
 - 1ul 20mg/ml glycogen (Roche 901393)
 - 122ul iced ethanol

5. Vortex to mix, centrifuge for 30min, 14,000rpm.
6. Remove supernatant and dry pellet (e.g. using the Speed Vac Concentrator (Savant)).
7. Add 10ul TE buffer (gives a concentration of 100ng/ul). Vortex and centrifuge briefly.
8. Leave at room temperature or 37°C for 2 hours with occasional vortexing and centrifugation to fully dissolve precipitate prior to storing at -20°C.

For Digoxigenin labelling, Biotin-11-dUTP and solution A4 were replaced with 5ul of Digoxigenin DNA labelling mix (Boehringer Mannheim, now Roche, 1277 065 (1mM dATP, dCTP, dGTP, 0.65mM dTTP, 0.35mM Dig-11-dUTP).

For direct labelling, Biotin-11-dUTP was replaced with 1ul of either fluorogreen or fluorored (Amersham).

If a probe was to be used frequently e.g. DG/VCFS and DMD cosmid, 100ul labelling mixes were prepared. Appropriate volumes of competitor and salmon sperm DNA were added after step 3, the DNA ethanol precipitated with 1/10 volume Sodium acetate pH5.2 and 2-3 volumes ethanol as above, and the pellet resuspended in hybridisation buffer rather than TE. The probe was then ready to aliquot and denature as required.

2.9 Oligonucleotide Primers

All non-commercial primers used in this study were prepared by the primer service in this department using an ABI 319 oligonucleotide synthesiser according to manufacturer's instructions.

2.9.1 Primer De-protection

All primers were supplied in a column and required to be de-protected before use.

Procedure

1. Collect 1ml of ammonium hydroxide (NH₃OH, Aldrich) in a 1ml polypropylene syringe and connect to one end of the column. Attach an empty syringe to the other end.
2. Gently push through the column, displacing the barrel of the second syringe. Ensure that the column is filled with liquid.
3. Push the NH₃OH back and forth through the column three times over a two to three hour period, ensuring the column is always liquid filled. Avoid formation of 'bubbles' in the column. The column should decolourise from yellow to white.
4. Withdraw the NH₃OH and expel into round-bottomed, screw-capped vials (Nunc).
5. Make up volume to ~2.0mls (full vial) by passing more NH₃OH through the column using a fresh syringe.
6. Tightly cap vial and incubate floating in a 55°C water bath overnight.

7. Store at -70°C till required.

2.9.2 Oligonucleotide Evaporation or Precipitation

De-protected oligonucleotides had to be removed from NH_3OH before use. This was achieved by one of two methods:

- A volume of primer (e.g. 0.45ml, leaving enough for three subsequent evaporations or precipitations) was precipitated with 1/10 3M Sodium acetate pH5.2 and 2-3 volumes of cold ethanol, centrifuged at 13,000rpm for 30min, vacuum dried and 500ul of sterile purified water added.
- A volume of primer (e.g. 0.45ml) in an open vial was left overnight in a fume hood to evaporate, then 500ul of sterile purified water added.

2.9.3 Assessment of Oligonucleotide Concentration

The absorbance of the primer DNA was obtained using a Gene Quant II RNA/DNA Calculator as described above. DNA concentration ($\mu\text{g/ml}$) was calculated by the spectrophotometer, using single-stranded DNA conversion factor 33 (1 unit of absorbance at 260nm = 33 μg single stranded DNA/ml).

The numbers of A, G, C and T bases in the primer were determined and the molar extinction coefficient of the primer calculated as:

The no. of A bases (16,000) + no. of G (12,000) + no. of C (7000) + no. of T (9,600).

The molar concentration of the primer stock (μM = pmoles/ μl) was then calculated as: (absorbance at 260nm x dilution factor) divided by the molar extinction coefficient.

2.9.4 Calculation of Primer Annealing Temperature

This uses the formula $4(\text{C}+\text{G})+2(\text{A}+\text{T})$ (range + or - 5°C). For example:

Factor IX 3'G (Cinti et al 1993 X1) C+G=11, A+T=9 so annealing temperature should be 62°C +/- 5°C

Factor IX 3'C (Cinti et al 1993 X2) C+G=10, A+T=11 so annealing temperature should be 62°C +/- 5°C

2.10 Preparation of Slide-Bound Target Material

Chromosome culture and harvest

Slide-bound metaphase chromosome spreads were made from fixed cell suspensions provided by the cytogenetics department of this Institute. On receipt, cell suspensions were transferred to 1.5ml microfuge tubes (Sarstedt) and stored at -20°C till required.

Slide preparation

1. Clean microscope slides by overnight soaking in Decon (Decon Laboratories, Hove, Sussex) and rinse with running tap water, first warm (for 30min) then cold (90min). The washed slides can be stored at 4°C in purified water for one week.
2. Centrifuge the vial of cell suspension at 1200rpm for 7min, remove the supernatant and discard. Re-suspend the pellet in enough fresh Carnoy's fixative (usually 200 to 500ul) to provide a slightly milky suspension.
3. Tilt a cold, clean wet slide on its long side and, using a Pasteur pipette, place two drops of cell suspension side by side near the slide edge. Allow the drops to run downwards before levelling the slide and air drying. The area of slide-bound material produced is easily enclosed by a 22mm² coverslip for FISH.
4. Before use evaluate the mitotic index and metaphase quality of the slide preparation by phase contrast light microscopy with a x10 objective lens.

2.11 Lipsol Banding (Malcolm et al 1981)

Staining solution

Add 3g Leishman powder to 2L methanol. Stir to dissolve then filter. For use, dilute 1 part stain in 3 parts buffer (pH6.8).

Procedure

1% Lipsol detergent (LIP Equipment and Services Ltd, Yorkshire), freshly made in purified water, was applied to 1 day-old slides for 20-25 seconds (or as required) then rinsed with saline. The slide was then flooded with Leishman stain for 2-3 minutes, rinsed with pH6.8 buffer and mounted in pH6.8 buffer using a 22 x 64mm coverslip.

2.12 Pre-Hybridisation Photography

(used prior to the availability of software-enhanced reverse DAPI banding)

2.12.1 Locating, Photographing and De-staining Slides

1. Locate complete, well spread and adequately banded metaphases within 20 Vernier units on the slide (representing ~2 cm²) and photograph them (Kodak Technical Pan 2415 film). Avoid spreads at slide edge. Note Vernier reading for each cell to allow re-location.
2. Wipe off excess immersion oil, remove coverslip by soaking in pH6.8 buffer, mark the area to be probed with a diamond pen and de-stain slide in an ethanol series.
3. Air-dry and use for ISH within 48 hours or store frozen in a box with dessicant.

4. Develop the film (to check photography has been successful) before ISH.

2.12.2 Developing Films

Solutions (Prepared fresh as required):

Developer - 1 part Kodak Dektol in 3 parts water

Stop buffer - 3% (48ml) glacial acetic acid in 1600ml water

Fixer - 1 part Ilford Hypam Fixer in 3 parts water

Procedure

1. In dark, remove film from camera, wind on to developing reel and place in developing tank.
2. Add in succession:
 - Developer, agitate every 30 seconds for 4.5 minutes then pour off.
 - Stop Buffer, agitate for 30 seconds then pour off.
 - Fixer, agitate every minute for 3.5 minutes then pour off.
3. Wash, under running cold tap water for at least ten minutes. Remove film from reel and hang to dry. Examine to ascertain negative image quality.

2.12.3 Printing Films

Following hybridisation, if signal appeared to be present, negatives were printed.

Procedure

1. Prepare trays containing:
 - Ilford paper developer, 1 part in 9 parts water.
 - Running cold water.
 - Ilford paper fixer, 1 part in 9 parts water.
2. For every negative, determine exposure time, expose paper and pass through developer, running water, fix and water again prior to drying.

2.13 Fluorescence *In Situ* Hybridisation and Detection of Liquid DNA Probes

2.13.1 FISH with Indirectly Labelled Non-Commercial DNA Probe

Adapted from Carter et al (1992).

Materials

50% Dextran sulphate (Sigma D-8906)

Add 2.5g Dextran sulphate to 3ml purified water. Allow time to 'wet' and mix frequently. Adjust volume to 5ml with purified water.

Hybridisation Buffer

- 5ml formamide (Fluka 47670) 50%
- 2ml 50% dextran sulphate 10%
- 0.5ml 20xSSC 1xSSC
- 2.5ml sterile water

Aliquot and store in microtubes at -20°C.

Sonicated salmon sperm DNA (10.4mg/ml Salmon Testes DNA Sigma D9156)

- Switch on sonicator (Soniprep 150, MSE) and allow to warm for 15min.
- Place DNA, on ice, under the sonicator probe. Close sonicator door. Wearing ear protection, sonicate at high power for four periods of 15 seconds.
- Boil vial for 10min.
- Check fragment size is 100-500bp by agarose gel electrophoresis.
- Store at 4°C.

Blocking solution and diluent for detection agents

15% human AB serum (Sigma S-7148, now discontinued) in 4XT. The amount required depends on the number of ISH areas to be detected but 5ml is usual. Alternatively, use 5% low fat milk powder in 4XT. Prepare 100ml, aliquot 5ml volumes, and store at -20°C.

Detection solutions

Volumes in brackets represent the amount of detection agent in each 100ul detection solution prepared. One ISH area requires 100ul of each appropriate solution per layer.

- FITC- or Texas red-avidin: FITC-avidin (Vector Labs A-2011) or Texas red-avidin (Jackson Immunoresearch 003-070-083) 5ug/ml in blocking solution (i.e. 0.25ul of 2mg/ml stock FITC- or Texas red-avidin in 99.75ul).
- Biotinylated anti-avidin: As FITC-avidin solution but replacing FITC-avidin with biotinylated anti-avidin (Vector Labs BA-0300).
- Mouse anti-digoxigenin FITC: Mouse anti-digoxigenin FITC (Sigma F3523) 0.3% in blocking solution (i.e. 0.3ul of mouse anti-digoxigenin FITC in 99.7ul).
- FITC anti-mouse: FITC anti-mouse (Sigma F3008) 0.5% in blocking solution (i.e. 0.5ul of anti-mouse FITC in 99.5ul).
- Mouse anti-digoxigenin FITC + biotinylated anti-avidin: Mouse anti-digoxigenin FITC 0.3%, biotinylated anti-avidin 5ug/ml in blocking solution (i.e. 0.3ul mouse anti-digoxigenin FITC and 0.25ul 2mg/ml biotinylated anti-avidin in 99.45ul).

- FITC anti-mouse + Texas red-avidin: FITC anti-mouse 0.5%, Texas red-avidin (Jackson ImmunoResearch 003-070-083) 5ug/ml in blocking solution (i.e. 0.5ul FITC anti-mouse and 0.25ul 2mg/ml Texas red-avidin in 99.25ul).

Procedure

Slide preparation

If banding was not performed, slide preparations were checked for mitotic index and quality using phase contrast microscopy. Suitable slide areas were marked with a diamond pencil and generally used for ISH within a few days of preparation. Prior to ISH, slides were dehydrated in an ethanol series (70%, 85%, 100%, 2min in each).

Slide denaturation

Slides were placed in a Coplin jar containing 70% formamide at 70-72°C for 2min. Transfer to ice-cold 70% ethanol for 2min arrested denaturation. Slides were again dehydrated in an ethanol series, and allowed to air dry.

Probe preparation and denaturation

A. If the probe was to be pre-annealed with competitor DNA (YACs, BACs, cosmids, larger plasmids of > ~6kb) the following volumes were added to a microfuge tube for each 22mm² slide area to be hybridised with 100ng of labelled probe:

- 1ul 100ng/ul labelled probe stock
- 1ul 10ug/ul total human genomic DNA (Cambio CA97205) or 2ul 1ug/ul Cot1 DNA (Gibco BRL 15279-011)
(concentration might be varied according to the amount of CISS required)
- 0.4ul 10ug/ul sonicated salmon sperm DNA (optional)

If probes were to be co-hybridised, an appropriate volume of the additional probe was also added.

Hybridisation buffer was added to make the volume 10ul unless the total DNA volume would comprise more than 30% of the hybridisation mix volume. In that case the DNAs were re-precipitated, as in nick translation, and the pellet dried before adding 10ul hybridisation buffer. Doubling the concentration of labelled probe prepared in TE (to 200ng/ul) could remove the need for this extra step. With the most frequently used probes, the entire labelling product was precipitated with competitor DNA immediately after nick translation and stored ready-to-use in hybridisation buffer.

The probe mix was denatured by 5 minute incubation at 70°C, then transferred to 37°C for 30-60 minutes to allow blocking of nonspecific, highly repetitive sequences.

B. If probe was not to be pre-annealed (plasmid probes phins310 and pK082 only):
0.5-1ul of 100ng/ul labelled probe and hybridisation buffer (to a 10ul volume) were added to a microfuge tube. This was denatured at 70°C for 5 minutes immediately before ISH.

Hybridisation

Probe mix was applied to marked slide area, covered with a glass coverslip and sealed with rubber solution. Slides were incubated on a metal tray floating in a water bath at 37-43°C (depending on probe) overnight (16 hours).

Post-hybridisation washing and detection

Subdued lighting was used and all washes were performed in Coplin jars.

1. Gum and coverslips were carefully removed and slides soaked for 5min (or less if reduced stringency required for smaller probes) at 42°C in each of two jars of pre-warmed 50% formamide. Alternatively, slides were washed in salt solution (usually 0.4xSSC, 72°C, 2min) and step 2 omitted.
2. Following 50% formamide, slides were washed twice in 2xSSC at 42°C (usually 5min each wash).
3. Appropriate detection solutions were prepared, incubated for 10min at room temperature then centrifuged for 10min at 14,000rpm to pellet any precipitate.
4. Slides were briefly soaked in 4XT. Blocking solution (100ul) was added to each ISH area then covered with Parafilm 'M' laboratory film (American National Can, Chicago). Slides were incubated at 37°C for 10min, by floating in a water bath on a metal tray.
5. If detecting biotinylated probe with FITC: 100ul FITC-avidin was added to the ISH area, covered with Parafilm and incubated for 15min at 37°C as before.

Detection agents for this first detection layer were varied as required:

If detecting DIG-labelled probe with FITC then mouse anti-digoxigenin FITC solution was substituted for FITC-avidin solution.

If performing dual hybridisation detecting DIG and biotin labelled probes then Texas red-avidin was used alone for the first layer.

6. Parafilm was removed and slides washed twice for 5min in 4XT at room temperature.
7. If detecting only a biotinylated probe, 100ul biotinylated anti-avidin solution was added to the ISH area, covered with Parafilm and incubated at 37°C for 15min, as before.
If detecting only a DIG-labelled probe then FITC anti-mouse solution was substituted and stages 9 to 11 omitted.
If performing dual hybridisation then mouse anti-digoxigenin FITC+biotinylated anti-avidin solution was used.

8. Parafilm was removed and slides washed twice for 5min in 4XT at room temperature.
9. If detecting biotinylated probe then slides were incubated again with 100ul FITC-avidin solution at 37°C for 15min as before.

If performing dual hybridisation, anti-mouse FITC+Texas red-avidin solution was used.

10. Parafilm was removed and slides washed twice for 5min in 4XT at room temperature.
11. If using a biotinylated probe requiring further amplification of signal, stages 7-9 were repeated.
12. Excess 4XT was drained and each slide mounted in 30ul of counterstain (DAPI or DAPI + PI as appropriate) overlayed with a 64x22mm glass coverslip. Slides were stored in the dark below 4°C.

2.13.2 FISH with Commercial DNA Probe

Biotinylated chromosome paint (Cambio) was prepared (5ul + 4.5ul hybridisation buffer + 0.5ul Cot1 DNA per 10ul hybridisation area) then denatured, pre-annealed, hybridised and detected as detailed in section 2.13.1.

Digoxigenin-labelled satellite DNA probes (ONCOR/Qbiogene) were prepared (0.5ul + 9.5ul hybridisation buffer) and also processed as above, but without pre-annealing.

Directly labelled probes were prepared and processed according to the manufacturer's instructions.

2.14 Fluorescence *In Situ* Hybridisation with Slide-Bound Cytocell Probes

2.14.1 Chromoprobe-T Procedure (adapted from the manufacturer's protocol)

1. Pre-heat 70% formamide in a Coplin jar to 72°C in a covered water bath.
2. Wash slide in 2xSSC for 2min at room temperature.
3. Dehydrate slide in an ethanol series (70%, 85%, 100%, 1min in each). Air dry.
4. Denature slide by immersing in 70% formamide for 2min at 72°C.
5. Quench in ice cold 70% ethanol for 2min and dehydrate in an ethanol series as above. Air dry.
6. Mix the provided hybridisation solution and pre-warm to 37°C for a minimum of 2min.
7. Working in subdued light, pre-warm to 37°C the slides for ISH and Chromoprobe-T coverslips (Teflon-coated side upwards) by placing on a hotplate for 2min.
8. Apply 10-15ul of pre-warmed hybridisation solution to each hybridisation area.

9. Place the Chromoprobe-T coverslip (Teflon side down) on the ISH area. Allow hybridisation solution to spread to the coverslip edges by applying gentle pressure.
10. Seal the coverslip edges with rubber solution (applied with a plastic syringe).
11. Leave on the 37°C hotplate for at least 5min.
12. Check, using the liquid crystal thermometer supplied with the kit, that the temperature of the denaturing hotplate is 74-75°C.
13. Ensuring slide is in good contact with the hotplate, denature for 2.5min (Cytocell recommend 2min) at 74-75°C.
14. Cool for 30 seconds on the bench at room temperature, place slide/Chromoprobe-T combination on a metal tray, and float tray in a covered 37°C water bath overnight.
15. Prepare 0.4xSSC in a Coplin jar and heat to 72°C in a covered water bath.
16. Remove the Chromoprobe-T (and gum) carefully from the slide and place slide in pre-heated 0.4xSSC for 2min, 72°C.
17. Place the slide in 4XT, room temperature, for 30 seconds. Drain off excess solution by placing slide briefly on its edge.
18. Apply 30ul DAPI/mountant then a glass coverslip (22x64mm) to slide.

2.14.2 Multiprobe-T Procedure (adapted from manufacturer's protocol)

1. Pre-heat 70% formamide in a Coplin jar to 72-74°C in a covered water bath.
2. Template slide spotting:
 - Soak template slide 2min in 100% methanol. Polish dry with a clean soft tissue.
 - Add ~150ul Carnoy's fixative to the pelleted metaphase preparation. Pipette 2ul of the cell suspension on to one slide area and allow to dry. Examine using phase contrast microscopy. If cell density is too high, dilute the suspension with more fix. If too low (require at least 5 metaphases in each template square) centrifuge it and add less fix (minimum 60ul). Placing an extra drop of fix on the 2ul spot during spreading or warming the slide over a 65°C water bath may reduce cytoplasm around metaphases.
 - Pipette 2ul on to all areas in the following order to prevent drops of suspension interfering with the spreading of adjacent drops -
 1st row odd numbers, 2nd row even numbers, 3rd row odd numbers then, after drying,
 1st row even numbers, 2nd row odd numbers, 3rd row even numbers.
3. As Chromoprobe-T steps 2 - 6.
4. Float a lidded plastic box (supplied) in a 37°C water bath.
5. In subdued light, warm Multiprobe device to 37°C (label side down) on a hotplate.
6. Apply 1ul of warmed hybridisation solution to each of the raised bosses of the device.

7. Carefully invert the template slide so that square 1 is located over the top right hand area of the device, marked in yellow. Lower the slide over the device, ensuring slide and device are properly aligned. Apply gentle pressure to ensure hybridisation buffer is spread to the edges of each Multiprobe area.
8. Lift the slide/Multiprobe-T device combination and invert so that the slide is underneath.
9. Leave at 37°C for 20min.
10. Check, using the liquid crystal thermometer supplied, that the temperature of the denaturing hotplate is 74-75°C.
11. Transfer the slide/Multiprobe-T to the 74-75°C hotplate taking care to hold it level. Ensure the slide is in good contact with the hotplate. Denature for 2-3min.
12. Cool for 30 seconds at room temperature on the bench. Place in the warmed plastic box, replace lid and float at 37°C overnight in a water bath with no lid.
13. Prepare wash solution (0.4xSSC) in a Coplin jar and heat to 72°C in a water bath.
14. Remove the device from the slide and place in pre-heated 0.4xSSC for 2min, 72°C.
15. As Chromoprobe-T steps 17-18.

2.15 Visualisation

Most slides were examined by epifluorescence using a Zeiss Axioskop microscope with appropriate optical filters (Table 2-1) and initially a 50W, then latterly a 100W, mercury vapour light source. Images were captured by a digital imaging system attached to this microscope (Fig.1-8). Rhodamine, FITC, and DAPI excitation filters were present on a Ludl filter wheel, and triple bandpass emission filter on a slider bar. A cooled CCD camera (Photometrics, Tucson, AZ, USA, Kodak KAF 1400 chip (1317x1035 pixels)) was controlled by a Macintosh computer (initially Quadra then Powermac) with image manipulation and analysis software (Smartcapture V2.1, Digital Scientific).

Breakpoint mapping experiments involving BAC and PAC clones were examined using a Zeiss Axioplan imaging microscope attached to a Cohu camera and Applied Imaging workstation with Cytovision software.

Prior to the availability of digital imaging, colour photomicrographs were taken using Kodak Ektachrome film (ASA 400) which was processed commercially.

2.16 Analysis Procedure

2.16.1 Using a Conventional Fluorescence Microscope

Early FISH Investigation of t(4;11) Family Members Using Plasmid Probes

Pre-banded and photographed, DAPI-stained hybridised cells were re-located using Zeiss filter combination 1. FITC signals on these post-ISH metaphases, appearing as yellow-green spots against the red PI counterstain using filter set 9, were marked on the relevant photographs. A record of signal distribution was prepared by marking on an ideogram the positions of all signals in all examined cells, and Chi square (χ^2) testing performed to determine the significance of any signal peaks (as in Malcolm et al 1981).

Early Work with DG/VCFS and DMD Cosmids

The required number of metaphases were examined (see appropriate Results sections) and the presence or absence of probe signals recorded. Target chromosomes were identified by control probes (as in DMD/BMD studies) or morphology.

2.16.2 Using Digital Imaging

Software enhanced reverse DAPI banding was used to identify the chromosomal localisation of signals in mapping studies. These could then be recorded directly on an ideogram and, if necessary, χ^2 testing performed to determine the significance of any signal peaks.

PI had previously been included in counterstain so that, with conventional fluorescence microscopy, the use of Zeiss filter 9 could allow green FITC signal to be viewed together with red chromosomes. However when imaging such preparations an interference filter had to be inserted on capturing the FITC signal in order to block simultaneous PI emission, and this movement during the capture process could introduce an image registration shift. PI was therefore omitted from the counterstain.

For microdeletion studies, the required number of metaphases were scored, recording the presence or absence of probe signals. Chromosomes were identified by control probes or software enhanced reverse DAPI banding. At least two typical metaphases from each case were archived. Images were stored on 940MB optical disks (Panasonic).

FLUOR	EXCITATION λ	EMISSION λ	FILTER SET
FITC	490	525	Zeiss 9, 10
Rhodamine	540-560	580	Zeiss 15
DAPI	345	425	Zeiss 1, 2
PI	520	610	Zeiss 9
Spectrum Green	505	535	Vysis Spectrum Green
Spectrum Orange	545-555	585	Vysis Spectrum Orange, Zeiss 15

Table 2-1 Optical filters.

2.17 Amplification of Max-Planck Institute 21-Specific YACs

Adapted from the supplied Max-Planck Institute (MPI) protocol.

To avoid artefacts caused by DNA contamination only autoclaved reagents were used.

Procedure

1. Add to a 0.5ml microfuge tube:

- 5.0ul 10xbuffer (Promega, magnesium free)
- 5.0ul 2.5mM dNTPs (Pharmacia, a 1/10 dilution of 25mM)
- 2.0ul 20pmol/ul DOP primer (a 1/10 dilution of 200pmol/ul stock, Telenius et al (1992))
- 5.0ul 25mM MgCl₂ (Promega)
- 30.6ul sterile ultrapure water

2. Leave microfuge cap open, UV-irradiate (Amplirad), 10min.

3. Add:

- 2ul (100ng/ul) YAC template (1st re-DOP reaction, supplied by MPI)
- 0.4ul (2U) Taq DNA Polymerase (Promega 5U/ul)
- 50ul mineral oil overlay

4. Close cap, transfer to thermal cycler (Perkin Elmer), and run PCR:

Denaturation at 94°C for 1min, followed by 94°C, 1min; 40°C, 1min; 72°C for 4min (35 cycles) then 72°C for 10min. Soak at 15°C.

5. After carefully removing 45ul through the oil, the product was either precipitated immediately or first stored at 4°C overnight.

6. To precipitate PCR product, add to 45ul of product in a 1ml microfuge tube and mix:

- 4.5ul 3M Sodium acetate pH5.2
- 1ul glycogen
- 125ul (2-3 volumes) ethanol (-20°C)

7. Place at -40°C, 15min.

8. Centrifuge at 13,000rpm (Eppendorf microfuge), 30min.

9. Vacuum dry (Speed Vac Concentrator).

10. Add 30ul TE buffer, pH8

11. Float in water bath at 37°C, 90min.

12. Measure absorbance at 260nm (section 2.7). One PCR amplification should yield 5-10ug/50ul according to MPI.

2.18 Agarose Gel Electrophoresis

Materials

Electrophoresis grade agarose (Gibco BRL 15510-027)

TBE buffer x10 (0.90M Tris, 0.9M Boric acid, 20mM EDTA):

108g Tris base

55g boric acid

40ml 0.5M EDTA

Make up to 1L in distilled water and dilute 1:10 for use.

Also available from National Diagnostics, Hull, England.

Loading mix:

0.25% Bromophenol blue (Sigma) in a 30% glycerol/70% water solution

1kb ladder mix:

10ul 1kb ladder (1ug/ul Gibco BRL 15615-016)

20ul loading mix

80ul TBE (or H₂O)

Store at 4°C or -20°C.

Procedure

1. Assemble electrophoresis system (EMBITECH RunOne).
2. Prepare a 1.3% agarose gel in a 250ml beaker:
 - 0.65g agarose
 - 50ml 1xTBE buffer
3. Heat to boiling in a microwave (about 1min).
4. When cooled to 60°C, add 3ul Ethidium bromide (10mg/ml aqueous solution, Sigma E1510, carcinogenic) in a fume hood.
5. Pour into gel tray with ends in place. Add comb and allow to cool for 20min.
6. Prepare samples: 5ul DNA plus 2ul loading mix.
7. Remove comb and ends from gel, cover with 1xTBE buffer.
8. Load samples. Load a size ladder (7ul) in one well.
9. Run at 100V (~45mA) until loading mix halfway down gel, remove gel and check using a UV light source (UV transilluminator) and gel documentation system (UVP).

2.19 Comparative Genomic Hybridisation Adapted from Kallioniemi et al (1994).

2.19.1 Slide Preparation

Only well spread, high mitotic index, low debris slide preparations of synchronised peripheral blood cultures from karyotypically normal male donors were selected for use as target chromosomes. Slides were generally prepared 1-3 days prior to CGH and stored at room temperature but could be frozen in a Parafilm-sealed box containing silica gel crystals (2-3 crystals in a small box with 2-3 slides) till required. Slides were not returned to freezer once removed.

Procedure

Target metaphase slides made from previously tested and stored fixed cell suspensions were examined prior to denaturation by phase contrast microscopy using a x10 objective, x10 eyepiece and x1.6 Optovar which provided the extra magnification to confirm the absence of unevenly-stained chromatids and damage on chromosomes (which could be frequent on Vysis slides). Dark, non-refractile chromosomes were preferred.

If, on checking slides after denaturation, chromosomes appeared pale and 'grey', another, less stringently denaturated slide would be examined prior to use in CGH. This effect was seldom apparent when metaphases of appropriate quality were selected for CGH.

Test DNA was generally hybridised to target metaphase preparations from more than one individual, increasing the chance of high quality signal being obtained in at least some of the hybridisation areas in an experiment.

2.19.2 Slide Dehydration and Denaturation

Procedure

1. Dehydrate in an ethanol series (70, 85, 100%, 2min each).
2. Denature in 70% formamide for 3min at 73-74°C.
3. Quench in ice cold 70% ethanol, 2min.
4. Dehydrate in an ethanol series (70, 85, 100%, 2min each).

2.19.3 Probe Labelling and Hybridisation

Most CGH investigations were performed using DNA extracted by the molecular genetics division of this Institute. Labelling was by nick translation, a modification of the method described for use of the Gibco BRL kit (18160-010).

Procedure

1. In 1ml microfuge tubes on ice prepare labelling mixes as below, using dUTP conjugated to a green fluor (Fluorescein-12-dUTP, NEN NEL413, or SpectrumGreen dUTP, Vysis 30-803200) for test DNA, and dUTP conjugated to a red fluor (Texas Red-5-dUTP, NEN NEL417, or SpectrumRed dUTP, Vysis 30-803400) for the control DNA. Mix after each addition.

• Kit dNTP mix (minus dTTP)	5ul
• DNA	1ug
• fluorophore labelled dUTP (green or red) 1mM	1ul
• DNA Polymerase 10U/ul (Promega M205A)	1ul
• Kit DNA Polymerase/DNAse I (Gibco BRL 18162-016)	5ul
• H ₂ O to bring final volume to 50ul	Xul

2. Incubate 15°C, 55min to obtain 500-2000bp fragments.

3. Place at 65°C, 10min to stop reaction.

4. Remove 10ul from each tube for later electrophoresis (to confirm fragment size). Store at -20°C.

5. To the test DNA tube add:

- 40ul (800ng) control DNA labelled as above or 2ul (200ng) Vysis labelled control DNA (female 32-804023 or male 32-804024).
- 30-40ul (40ug) Cot1 DNA. Use 20ul if employing Vysis labelled control DNA.
- 12ul 3M Sodium acetate pH5.2 (filter sterilised, Sigma S-7899) or 6.3ul if using Vysis labelled control DNA.
- 300ul iced ethanol or 170ul if using Vysis labelled control DNA.
- Mix, place at -70 °C, 15min.

6. Microfuge 14000rpm, 30min.

7. Remove supernatant and vacuum-dry pellet for 20min. The dry pellet must not be left standing, as fluorophores may decay faster when dry.

8. Add 10ul hybridisation buffer. Mix gently.

9. Denature in a water bath at 70°C, 5min.

10. Apply probe to marked hybridisation area on slide.

11. Hybridise at 39°C for 72 hours.

12. Perform the following washes in very subdued light:

- 50% formamide, 7.5min, 45°C (twice)
- 2xSSC, 5min, 45°C (twice)
- 4XT, 5min, room temperature

13. Dehydrate in an ethanol series as before.

14. Mount in 30ul Citifluor AF1 containing 1.6ug/ml DAPI.

2.19.4 Slide Examination and Image Processing

Procedure

1. Select for analysis metaphase spreads with fairly bright and even signals, bright DAPI counterstain and 'blocked' centromeres (no signal).
2. Record digital fluorescent images of all 3 colours with the CCD camera and Smartcapture software using automatic setting, gain 4. Ensure lamp is centred and focussed to achieve homogeneous illumination of the optical field.
3. Save and (following reverse-DAPI banding with Smartcapture software if necessary) process at least 5-10 of the highest quality images according to Quips CGH Karyotyper and Interpreter software by following the manufacturer's instructions (Vysis). Ignore signals on acrocentric short arms, centromeric and heterochromatic regions. Particularly note any signal which is visible on two apparently homologous chromosomes. Early CGH experiments were alternatively, or additionally, software processed using Smartcapture 'global' analysis according to manufacturer's instructions.

2.19.5 CGH Quality Assessment

- Ascertain even paint coverage, bright signal and 'blocked' centromeric regions.
- Examine images by eye - it may be possible to visualise an apparent imbalance on both chromosome homologues, helping to confirm its significance.
- Check for detection of the pseudoautosomal region on Xp (~2.6Mb) with relaxed threshold.
- A normal male versus normal female control CGH can be included. This should show a loss of entire X and gain of entire Y at thresholds 0.8 /1.2 without introducing false positive gains or losses (Kallioniemi et al 1994).
- Use test and control DNAs of differing gender to provide an internal control via the sex chromosomes (Weiss et al 1999). With female test and reference male DNA, gain should be visible on chromosome X unless threshold set to ≥ 2.0 . With male test/female reference, apparent loss should be visible unless threshold set to ≤ 0.5 .
- If the limits of 95% or 99% CI of test versus reference CGH ratio profiles are used as thresholds, gains or losses are identified when the 95% or 99% CI at the site of a suspected imbalance does not contain 1.0 (Weiss et al 1999).

- Repeat or duplicate CGH using a different control DNA. Ascertain that any apparent gains/losses are consistent.
- Repeat CGH with inverse labelling of test and reference DNA can be used to check that any apparent gains/losses are consistent (Kallioniemi et al 1994).
- Use FISH, if appropriate probes available, to confirm apparent imbalances and provide increased resolution.

2.20 Primed *In Situ* Labelling

2.20.1 Commercial Repetitive Target Sequence Primers and Kits

2.20.1.1 The PRINS Reaction Set

Initial experiments employed the PRINS Reaction Set (Boehringer Mannheim 1695932) with a human chromosome 12 alpha satellite-specific primer (1696009). The contents of the reaction mix and recommended denaturation and annealing/extension temperatures used were identical to those in test 1, Table 3-28.

Materials

Contents of PRINS Reaction Set, stored at -20°C:

PRINS labelling mix x10 (500uM of each of dATP, dCTP, dGTP, 50uM DIG-11-dUTP in 50% glycerol)

dTTP solution (450uM dTTP)

PRINS reaction buffer x10

Anti-digoxigenin-fluorescein Fab fragments (200ug/ml). Aliquot and store 'in use' vial for up to 2 months at 4°C. To prepare detection solution dilute 1:1000 in PBS containing 1% blocking solution (10% blocking reagent - available separately from Boehringer Mannheim - w/v in buffer comprising 100mM maleic acid, 150mM NaCl, pH7.5).

Additional reagents not provided in kit:

Taq DNA Polymerase 1U/ul (Boehringer Mannheim)

PRINS oligonucleotide primer (chromosome 12-specific, Boehringer Mannheim 1696009)

Wash buffer (0.2% Tween 20 in PBS)

PRINS stop buffer (0.05M NaCl, 0.05M Na₂EDTA, pH8, Gosden and Hanratty 1993) -

0.5ml 5M NaCl

5ml 0.5M Na₂EDTA

40ml purified water

Procedure (essentially, apart from counterstaining, as in manufacturer's instructions)

1. For each PRINS area add the following reagents to a sterile microfuge tube on ice:
 - sterile ultrapure water 13.5ul
 - PRINS reaction buffer x10 3.0ul
 - PRINS labelling mix x10 3.0ul
 - dTTP solution 3.0ul
 - oligonucleotide primer 5.0ul
 - Taq DNA polymerase (1U/ul) 2.5ul
2. Mark 22x22cm reaction areas on dry slides with fixed chromosomes. Place on a heating block and incubate at 94°C for 1min.
3. Add 25ul of the reaction mix and cover with a 22x22cm coverslip.
4. Incubate at 91-94°C for 3min to denature the chromosomal DNA (set the heating block to 95°C to obtain this temperature on the slide).
5. Adjust the heating block to 60°C and incubate the slides for 30min at 60°C.
6. Remove the slides from the block and stop the reaction by washing slides immediately for 5min at 60°C in pre-warmed stop buffer in a Coplin jar.
7. Wash three times for 5min at 37°C in wash buffer and once for 1min in PBS.
8. Incubate the slides in detection solution for 30min at 37°C in the dark.
9. Repeat step 7.
10. Mount and counterstain in DAPI or DAPI + PI in Citifluor AF1.

2.20.1.2 The Chromosome Print Kit

The Chromosome Print Kit (a commercial PRINS system available from Advanced Biotechnologies, Surrey) was later assessed. A programmable heat block (Omnigene, Hybaid), which compensated for the temperature difference between the block and the surface of the slide, was available for use by that time (Fig.3-25). Omnigene hot blocks also have an internal lid and reservoir which can provide a humid environment.

Materials

Contents of Print Kit:

Chromosome Print labelling mix, stored at -20°C

Chromosome Print oligonucleotide, stored at -20°C

Chromosome Print stop solution, stored at 4°C. Pre-warm 50ml to 65°C in a Coplin jar before use.

Chromosome Print wash solution x5, stored at 4°C. Add contents to 400ml sterile purified water and mix. Pre-warm to 45°C before use.

Blocking reagent, stored at 4°C. To prepare blocking buffer, add 1ml of pre-warmed wash solution and dissolve by vortex mixing.

Additional reagents not provided in kit:

CP-CPP Chromosome Print Polymerase 5U/ul

(Alternative: AmpliTaq /S Polymerase 20U/ul and MgCl₂ 25mM (Perkin Elmer))

Biotin-16-dUTP 1mM (Boehringer Mannheim 1093070)

(Alternatives: Digoxigenin label mix 1mM dATP/dCTP/dGTP, 0.65mM dTTP, 0.35mM dUTP-DIG (Boehringer Mannheim) or DIG-11-dUTP 1mM (Boehringer Mannheim))

Fluorescein Avidin DCS (Vector Laboratories, A-2001) diluted 1:500 in blocking buffer for use (0.25ul in 125ul blocking buffer)

Procedure (see Table 3-27)

1. For each PRINS area add the following reagents to a sterile microfuge tube on ice.

Either:

- | | |
|---|--------|
| • Chromosome Print labelling mix | 43.0ul |
| • Chromosome-specific primer | 5.0ul |
| • Chromosome Print polymerase | 1.0ul |
| • 1 mM Biotin-16-dUTP/digoxigenin-11-dUTP | 1.0ul |

Or:

- | | |
|--|--------|
| • Chromosome-specific primer | 5.0ul |
| • Digoxigenin label mix x10 | 5.0ul |
| • PCR Buffer x10 (Perkin Elmer) | 5.0ul |
| • MgCl ₂ (25mM, Perkin Elmer) | 2.0ul |
| • Sterile ultrapure water | 32.8ul |
| • AmpliTaq /S Polymerase 20U/ul | 0.25ul |

For experiment 4 the total volume of the reaction mix was adjusted to 30 rather than 50ul and the AmpliTaq /S concentration increased to 0.13U/ul (0.2ul/30ul). The concentrations of the other components were as above.

2. Mark 22x32cm reaction areas on 1 day-old slide-bound chromosome preparations.

Dehydrate in an ethanol series (70%, 85%, 100%, 2min each). Air dry. Add reaction mix and cover with a 22x32cm coverslip. Seal coverslip with rubber solution and allow to dry.

3. Place on a heating block pre-heated to 93°C and run program:

93°C for 1min, 60°C for 10min and 72°C for 15min.

4. Quickly remove coverslip and place slide in a Coplin jar containing pre-warmed stop solution. Incubate at 65°C, 1min.
5. For biotin detection:
 - Place slide in wash solution.
 - Prepare 40ul avidin-FITC detection solution for each slide reaction area.
 - Remove the slide from wash solution, blot the slide edges to drain. Add 40ul of blocking buffer, cover with Parafilm and incubate for 5min at room temperature.
 - Remove Parafilm, blot slide edges to drain buffer and add 40ul of diluted avidin-FITC. Incubate at 37°C, 30min, floating on a metal tray in a covered water bath.
 - Remove Parafilm and wash three times for 2min at 45°C in wash solution.Alternatively, for digoxigenin detection:
 - Place slide in 4XT at room temperature.
 - Block in 4XT + 15% human AB serum for 10min and detect DIG label as for FISH.
6. Mount and counterstain in DAPI or DAPI + PI in Citifluor AF1.

2.20.2 PRINS and Cycling PRINS Using 'In-House' Primers

Slides were processed using, as detailed in Tables 3-28, 30, 32, 35, 37, 38, 39, variations of the procedures and reagents described above. Reaction mixes were 30 or 50ul volumes, generally applied under 22x22mm or 22x32mm coverslips. Slides which were denatured in 70% formamide (for 2min at 70°C, unless otherwise stated) were (as in FISH) dehydrated in an ethanol series before and after denaturation. Label detection, generally following incubation in stop buffer (0.05M NaCl, 0.05M Na₂EDTA, pH8) for 5min at 60°C, was as described for FISH.

2.20.3 Ligase Pretreatment

Some slides were treated with T4 DNA Ligase to attempt to reduce background arising from nonspecific initiation at single strand nicks in the chromosomal DNA.

Materials

Ligase mix:

0.5ul T4 DNA Ligase (1U/ul, Gibco BRL)

2ul Ligase buffer (x5, Gibco BRL)

7.5ul sterile purified water

Ligase stop solution (0.5M NaCl, 0.05M Na₂EDTA, Gosden and Hanratty 1993):

5ml 5M NaCl

5ml 0.5M Na₂EDTA

40ml purified water

Procedure

1. Prepare ligase mix. Require 0.5U ligase in 10ul for each 22x22mm reaction area.
2. Place 10ul ligase mix on each reaction area, cover with a 22x22mm coverslip.
3. Place on a thermal cycler block in a humid chamber at room temperature for 1 hour.
4. Terminate the ligase reaction by soaking for 5min in stop solution.
5. Dehydrate slides in an ethanol series and air dry.

2.20.4 Fix Pretreatment

Place slide in Coplin jar containing Carnoy's fix for 45-60min. Remove and air dry.

Incubate in 2xSSC for 30min at 37°C. Dehydrate in an ethanol series.

2.20.5 Tyramide Signal Amplification

Materials

Contents of TSA-Indirect Kit (NEL730A, DuPont NEN), stored at 4°C:

Streptavidin-HRP (horseradish peroxidase) Conjugate 100ml

DuPont Blocking Reagent 3g

Biotinyl Tyramide sufficient for 50 slides

Reconstitute with 0.5ml of 100% ethanol. Close immediately after use to avoid volatility of ethanol affecting tyramide concentration.

Amplification Diluent (2x) 20ml

Diluted 1:1 with purified water for use.

Purchased separately:

Anti-Digoxigenin-POD Fab fragments (Horseradish peroxidase conjugate) 150U/ml when reconstituted (Boehringer/Roche 1207733). Dilute 1:50 or 1:100 in TNB (see below) for use. Prepare 100ul per slide.

Streptavidin-Fluorescein 1ml, 1mg/ml (NEL720). Dilute 1:500 in TNB (1ul + 499ul TNB). Prepare 100ul per slide.

Reagent Preparation

Biotinyl Tyramide solution:

Prepare a 1:50 dilution of Biotinyl Tyramide stock in 1x Amplification Diluent.
Approximately 300ul per slide required (6ul Biotinyl Tyramide + 294ul Diluent).

TNT Buffer (0.1M Tris-HCl pH7.5, 0.15M NaCl, 0.05% Tween 20):

1M Tris-HCl pH7.5	60ml
3M NaCl	30ml
Purified water	510ml
Tween 20	300ul

TNB Buffer (0.1M Tris-HCl pH7.5, 0.15M NaCl, 0.5% DuPont Blocking Reagent)

Mix in a 20ml Universal container (Sterilin):

1M Tris-HCl pH7.5	1.0ml
3M NaCl	0.5ml
DuPont Blocking Reagent	0.05g
Purified water	to 10.0ml

Heat at 65°C for 1 hour to dissolve blocking agent. May store for 1 month at -20°C.

Procedure

Digoxigenin label was used in the PRINS or FISH prior to tyramide amplification. Stop buffer was omitted following PRINS on most occasions (to avoid TSA inhibition, verbal communication, DuPont NEN).

The method used was essentially that recommended by the manufacturer, with some modifications while attempting optimisation.

For evaluation of amplification effect, control slides were included which were also blocked with TNB and washed with TNT buffer but detected using anti-digoxigenin FITC and anti-mouse FITC (though with intermediate TNT rather than 4XT washes) as in standard FISH.

1. Following post-ISH washes or PRINS, wash slide three times for 5min in TNT buffer at room temperature, with agitation.
2. Add 100ul TNB Buffer to each FISH/PRINS area and cover with Parafilm.
3. Incubate at 37°C for 30min floating on a metal tray in a water bath.
4. Drain off TNB Buffer, add 100ul of Anti-Digoxigenin-POD diluted in TNB Buffer (1:50 or 1:100) and cover with Parafilm.
5. Incubate at 37°C for 30min floating on a metal tray in a water bath.
6. Wash slide three times for 5min in TNT buffer, with agitation.
7. Add 300ul of 1:50 dilution of Biotinyl Tyramide stock in 1x Amplification Diluent and incubate at room temperature for 7 or 10min (manufacturer suggests 3-10min).

8. Wash slide three times for 5min in TNT buffer, with agitation.
9. Add 100ul of a 1:500 dilution of Streptavidin-Fluorescein conjugate in TNB Buffer.
10. Incubate at 37°C, 20-30min, floating on a metal tray in a water bath.
11. Wash slide three times for 5min in TNT buffer, with agitation.
12. Counterstain and mount in Citifluor AF1 with DAPI or DAPI + PI as for conventional FISH. Evaluate using fluorescence microscopy with appropriate optical filters.

NEN suggest reducing anti-hapten antibody concentration, labelled probe concentration and time in Streptavidin-Fluorescein if signal overly bright and lacks resolution.

2.20.6 PCR

PCR in solution was performed to assess the amplification efficiency of some PRINS primers and reaction mixes. The variety of reagents and conditions used are described in Tables 3-29, 31, 33, 34, 36. Reaction mix (50ul) in 0.5ul microtubes was overlaid with mineral oil, the tubes placed in a thermal cycler (Perkin Elmer) and the PCR programme commenced.

Agarose gel electrophoresis of the PCR products was performed (see section 2.18) and the results photographed using a UV transilluminator and gel documentation system (UVP).

2.21 Proteinase K Pretreatment (broadly according to Kallioniemi et al 1994)

Materials

0.2ug/ml Proteinase K (Sigma P2308) in 20mM Tris-HCl, 2mM CaCl₂ pH7.5. Aliquot and store at -20°C.

Procedure

If pretreating CGH slides, first dehydrate and denature as usual.

Place 200ul 0.2ug/ml proteinase K on the slide, cover with a glass coverslip and incubate for 3min (PRINS) or 7.5min (CGH) floating on a metal tray in a water bath at 37°C.

Remove coverslip and dehydrate in an ethanol series prior to PRINS (may first treat with ligase) or continuing CGH. Before proceeding, examine slide using phase contrast microscopy to ensure metaphase spreads have not been damaged.

3. RESULTS

3.1 Chromosomal Localisation of Unique Sequences and Breakpoint Mapping

Following several years experience of chromosomal localisation using chromogenic ISH (publications included Morrison et al 1990, 1991a,b, 1992), fluorescence detection of ISH preparations was introduced to this department in 1992. A period of considerable development followed during which several genes were localised and modifications to the methodology were introduced (Murphy et al 1993, Morrison et al 1994a,b, McBride et al 1995a,b) with the aim of rendering the process more rapid and improving accuracy, reliability and sensitivity. Most notable was the digital recording of FISH images, which allowed software-enhanced reversed DAPI banding and therefore chromosome identification directly from the computer screen. This, and the availability of larger clone inserts for mapping, obviated time consuming pre-hybridisation banding and photography for chromosome identification and statistical analysis of signal distributions.

Mapping, in collaboration with a number of research groups, continued during the course of this study (1996 onwards). The research groups have been listed, together with probe details, in Appendix I and a chronologically ordered summary of the mapping results obtained is presented in Table 3-1.

Probes from these researchers were generally supplied ready for assessment of DNA concentration and incorporation of labelled nucleotide by nick translation. FISH mapping was executed on metaphase spreads prepared from the synchronised cultured lymphocytes of at least two karyotypically normal human males, more precise localisation involving selection of longer prometaphase spreads in these cultures. Initial mapping of clones was carried out with no knowledge of any previous chromosomal localisation results.

An obvious advantage of chromosomal localisation is that mapped DNA sequences may then be utilised in the characterisation of chromosome rearrangements. Chromosome 21-specific YACs and chromosome 8 and 21 BACs and PACs were sourced for breakpoint mapping investigations on fixed cytogenetic preparations from two patients with chromosomal rearrangements. These probes respectively required PCR amplification or culture and extraction prior to labelling. Their use in breakpoint mapping followed validation of probe map positions on karyotypically normal metaphase spreads. Unless stated, detection of all FISH localisations employed the biotin-avidin-FITC system, amplified at least once with biotinylated anti-avidin and another layer of avidin-FITC, and

a hybridisation temperature of 37°C was utilised to reduce stringency. Probe concentration, hybridisation and detection conditions used for each probe are recorded in Table 3-2. These were established following preliminary experiments with each probe to determine the conditions which would maximise signal strength and minimise nonspecific background.

CLONE	LOCUS NAME	SIZE (kb)	VECTOR	DESCRIPTION	RESULT OF MAPPING (BAND LOCATION)
1. PAC227		100-300	PAC	Endothelin-like sequence from HGM program PAC library.	11p12~p13
2. 82-h2	<i>MRCKβ</i> or <i>CDC42BPB</i>	100-300	PAC	Isolated from RP1 library by hybridisation to <i>CDC42BPB</i> 5'EST. Contains 460bp from exon1.	14q32.3
3. ? <i>hTERT</i>		70-100	P1	Putative telomerase protein component.	10q11.21~q11.22 and 10q22.3
518C13	<i>hTERT</i>	~180	BAC		5p15.33
4. 9;17; 22;30	? <i>SCD2</i>	~35-45	cosmid	Putative stearoyl coenzyme A desaturase 2	4q21.2~q21.3.
5. 925h10 937e12 949b09 856h04 858e10 759d03	21q-specific		PCR amplified YAC inserts	YACs from CEPH library.	21q21.1~q21.2 21q11.2~q21.1 21q11.2 21q11.2~q21.1 21q11.2 21q11.2
268F23 280N08 65E08 184E23 141D02	21q-specific		BAC	Clones from RP11 library.	21q11.2~q21.1 (21q21.1) 21q11.2~q21.1 (21q21.1) 21q11.2~q21.1 (21q21.1) 21q11.2~q21.1 (21q21.1) 21q11.2~q21.1 (21q21.1)
152M24	21q-specific		PAC	Clone from RP1 library.	21q11.2~q21.1 (21q21.1)
6. 369E15 177H13 395I14 14I17 138J2 356F24 263C6 564K10 527N22 350N15 44K6 51K12 198M21 231D20 137L15	8p-specific	~180	BAC	Clones from RP11 library.	8p21 (8p21.3) 8p21 (8p21.2) 8p21 (8p21.2) 8p21 (8p21.2) 8p21 (8p21.2) 8p21 (8p21.2) 8p12 (8p12) 8p12 (8p12) 8p12 (8p12) 8p11.2~p12 (8p12) 8p11.1~p11.2 (8p11.21) 8p11.1~p11.2 (8p11.21) 8p11.1~p11.2 (8p11.21) 8p11.1~p11.2 (8p11.21) 8q11.21~q11.22 (8q11.21)
	8q-specific	100-300	PAC	Clone from RP1 library.	

Table 3-1 Clones supplied for mapping and results obtained.

Chromosome 8 and 21 clones were only roughly localised, for identity confirmation. Their Ensembl localisation is bracketed.

PROBE	LABEL	HYBRIDISED PROBE CONCENTRATION ng/ul	BLOCKING AGENT (DETECTION)	CISS	DETECTION SYSTEM	NUMBER OF FLUOR LAYERS	POST-ISH WASH in F (50% formamide) or SSC
PAC227	BIOTIN	24	AB serum	0.8ug/ul total human DNA 30min	AVIDIN-FITC	3	2x5min F 42°C
82-h2	BIOTIN	8 to 10	AB serum	1ug/ul total human DNA 60min	AVIDIN-FITC	2 or 3	5min 2xSSC 72°C
putative telomerase protein component	BIOTIN	20	AB serum	0.5ug/ul Cot1 DNA 60min	AVIDIN-FITC	2	2x10min F 42°C
518C13	BIOTIN	5	AB serum	0.5ug/ul Cot1 DNA 60min	AVIDIN-FITC	2	2x10min F 42°C
	DIG	10	AB serum	0.5ug/ul Cot1 DNA 60min	anti-DIG-FITC	2	2x10min F 42°C
SCD2 9,17,22,30	BIOTIN	10	AB serum	1ug/ul total human DNA 60min	AVIDIN-FITC	2	2min 0.4xSSC 72°C
21q SPECIFIC YACs	TABLE 3-4	TABLE 3-4	AB serum	TABLE 3-4	TABLE 3-4	1 or 2	2x5min F 42°C or 2min 0.4xSSC 72°C
21q SPECIFIC BACs&PAC	BIOTIN	8	dried milk	1-2 ug/ul total human DNA 60min	AVIDIN-FITC	2	2x5min F 42°C
8p and 8q SPECIFIC BACs&PAC	BIOTIN &/or DIG	6 or 10 (dual ISH)	dried milk	1ug/ul total human DNA or 1.5ug/ul total human DNA (dual ISH) 60min	AVIDIN-FITC or AVIDIN-TEXAS RED/ anti-DIG-FITC	2	2x5min F 42°C or 2min 0.4xSSC 72°C

3.1.1 Endothelin 3-like Sequence

PAC227 had been isolated during a study of the role of endothelin 3 in diabetic retinopathy. It was supplied for chromosome assignment to determine if its location would match that of the given map position for endothelin 3 (20q13.2~q13.3, MIM 131242) and hence support its identification as an endothelin sequence.

Probe concentration was determined to be 1.05ug/ul prior to biotinylation by nick translation. Preliminary CISS hybridisations established optimal competitor concentration (0.8ug/ul total human genomic DNA in pre-annealing for a reduced period of 30 minutes), probe concentration (24ng/ul) and wash conditions (two 5 minute incubations in 50% formamide). Three layers of avidin-FITC were used in detection and human AB serum in 4XT was used as blocking solution.

After counterstaining with DAPI alone to avoid the image registration difficulties encountered when using PI (see Methods) post-ISH metaphases were analysed directly from the screen using digitally-enhanced DAPI bands for chromosome identification. Twelve metaphase chromosome spreads were analysed. Signal was visible only on chromosome 11, at the junction of bands p12 and p13, and was present on both homologues of chromosome 11 in 50% of cells (Fig.3-1).

3.1.2 Myotonic dystrophy protein kinase-related Cdc42-binding kinase β (*MRCK β*)

In the course of general characterisation of the human *MRCK β* sequence our collaborators had mapped it to 14q32.1~q32.3 using *MRCK β* -specific oligonucleotides to screen radiation hybrid panels. To verify this assignment a PAC clone of the human homologue of *MRCK β* (known as *CDC42BPB* or 82-h2) was isolated from the RP1 library and provided for chromosome localisation by FISH.

The concentration of 82-h2 supplied was 1ug/ul. Preliminary hybridisations of biotinylated 82-h2 established optimal competitor concentration (1ug/ul total human genomic DNA) probe concentration (8-10ng/ul) and wash conditions. Satisfactory results were obtained using post-ISH immersion in 2xSSC at 72°C for 5 minutes, which removed the requirement for formamide washes and the risk associated with their use. The use of only one FITC amplification did produce signal with this large sequence, but signal was easier to visualise without imaging following two rounds of amplification.

Twenty metaphase chromosome spreads were analysed. Signal was seen only on both homologues of chromosome 14, at band 14q32.3, in every cell (Fig.3-2, Moncrieff et al 1999). More refined mapping to 14q32.31~q32.32 was achieved by examination of the

ten longest metaphases (some with approximately 850 bands). Twelve of the signals on twenty chromosome 14 homologues were located at 14q32.31 and eight at q32.32.

3.1.3 Human Telomerase Reverse Transcriptase (*hTERT*, MIM 187270)

Our collaborators were involved in the isolation of a probe for the reverse transcriptase catalytic subunit of telomerase which would be suitable for FISH. This probe would allow direct physical mapping of the gene and additionally be a useful tool in their investigation of *hTERT* copy number in cell lines and tumours.

Initially FISH mapping was performed with a P1 clone containing a putative *hTERT* sequence. Its localisation to 10q11.21~11.22, with a weakly hybridising secondary site at 10q22.3, suggested it could not be *hTERT*, as *hTERT* had previously been tentatively mapped to distal chromosome 5p by PCR analysis of hybrid panels (Meyerson et al 1997). An *hTERT* BAC probe was therefore subsequently isolated by our collaborators and submitted for chromosomal assignment.

Two preparations of this probe, 518C13, were provided. One was biotinylated and the other (concentration 3 μ g/ μ l) was used for DIG labelling. The biotinylated 518C13 was hybridised to metaphase chromosome spreads at a concentration of 5ng/ μ l with 0.5 μ g/ μ l Cot1 DNA as competitor. Post-ISH washing involved two 10 minute incubations at 42°C in 50% formamide, and two layers of avidin-FITC were used in detection.

Only metaphase spreads with clear bands at 5p15.2 were analysed. Signal was present only on both chromosomes 5, at bands p15.32~p15.33, in all cells examined.

Biotinylated 518C13 was also co-hybridised with DIG-labelled 5p15.2-specific sequence (D5S23, Oncor Inc., Gaithersburg, MD). All ten metaphases examined showed 518C13 signal in the distal 5p15.3 region and distal to 5p15.2 signal on both chromosome 5 homologues.

DIG-labelled 518C13 (10ng/ μ l) was co-hybridised with biotinylated cri du chat region probe (5p15.3, Oncor), ascertaining that the cri du chat probe localised to proximal 5p15.3 and confirming that 518C13 lay distal to this locus (Fig.3-3).

As 518C13 appeared to have a very distal location on chromosome 5p, it was then co-hybridised with Chromoprobe-T 5ptel/5qtel probe combination (Cytocell, Adderbury, Oxfordshire), no individual 5ptel probe being commercially available, to determine if 518C13 could be ordered relative to the telomeric sequence. Biotinylated 518C13 (5ng/ μ l) in hybridisation buffer was used in place of the hybridisation buffer supplied with the coverslip-bound telomere probes. Formamide post-ISH washes were performed as for 518C13 single hybridisation and dual colour signal detection carried out with Cytocell

detection agents. All twenty metaphases examined, with chromosomes in the 550-850 band range, showed apparent co-localisation of 518C13 and the 5ptel cosmid 114j18. This confirmed 518C13 location to 5p15.33 and suggested that the sequences are no more than 1Mb apart. Sequence 114j18 was considered, by Cytocell, to lie within 300kb of the telomere associated repeats (these repeats may extend for several hundred kb, Knight and Flint 2000).

To ascertain that the 5ptel probe signal was, as expected, more distally located than 518C13, interphase analysis was attempted following the co-hybridisation of biotinylated 518C13, 5p15.2-specific probe (Oncor) and 5ptel/5qtel probe combination (Cytocell) visualised using Cytocell detection agents. As confirmed on control metaphase spreads, the red 5qtel probe signal was fortuitously consistently weaker than the red 518C13 signal and could be removed, by adjusting the colour intensity, for analysis. Although it was realised that tertiary structures in nuclei could interfere with reliable interphase ordering above 1Mb (Trask et al 1991) and that the 5p15.2 sequence would lie more than 1Mb from 518C13, no other distal 5p sequence was then commercially available, the 5p15.3 probe used in earlier FISH having been discontinued.

Only nuclei with three signals in a straight line (one signal distant from the other two) and with no doublet signals (which could be misinterpreted as two single signals of the same colour) were scored. Of thirty nuclei examined, seventeen met these criteria. All seventeen showed co-localisation of a green (presumably 5ptel) and red (518C13) signal, with a green 5p15.2 signal separate from this combination. Nuclear scoring was discontinued as it became obvious that the distance between the 5p15.2 signal and the other sequences was complicating analysis (signals were often in a curved line, for example). This investigation did, however, appear to suggest that the distance between the 5ptel sequence and 518C13 might be as little as 50kb - the lower limit of resolution for interphase analysis (Trask et al 1991).

In summary, these experiments confirmed the mapping of *hTERT* to the distal short arm of chromosome 5 and refined this localisation to band 5p15.33, distal to the commercial 5p15.3 cri du chat probe sequence (Oncor) and within 1Mb of subtelomeric sequence 114j18.

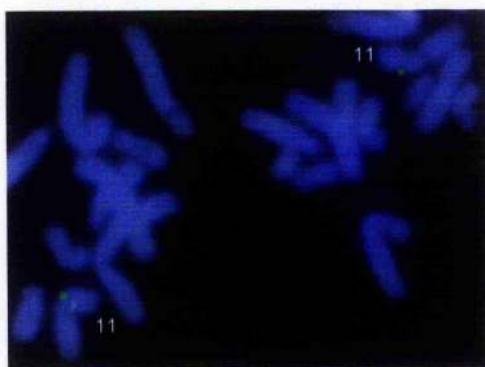


Fig.3-1 Localisation of PAC227 Endothelin-like sequence to 11p12~p13. Image on right shows reversed DAPI image used for chromosome identification.



Fig.3-2 Localisation of *MRCKβ* to 14q32.31~q32.32. Image on right shows reversed DAPI image with *MRCKβ* signal (green) superimposed.

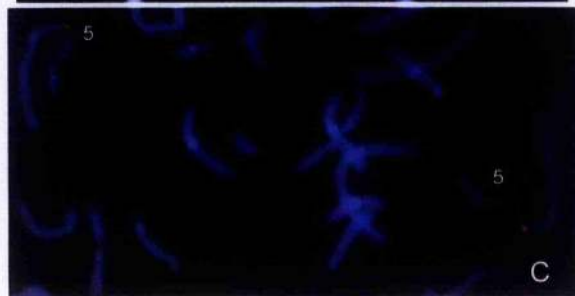
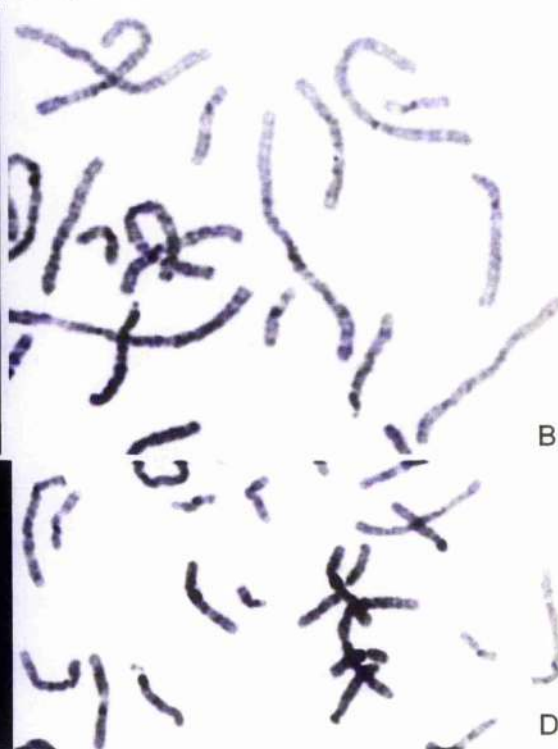
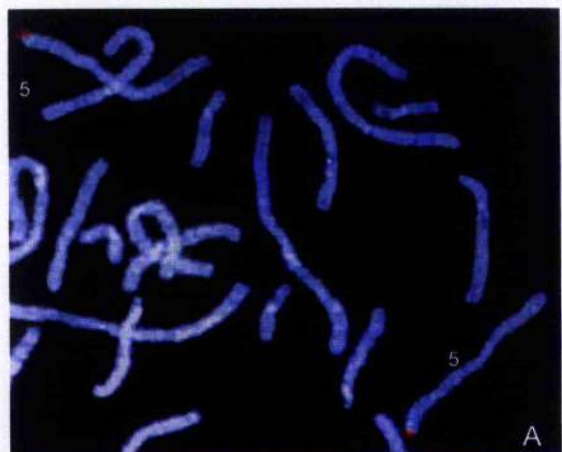


Fig.3-3 Localisation of *hTERT* to 5p15.33.

A: *hTERT* (red) co-hybridisation with 5p15.3-specific cri du chat probe (green, Oncor).

B: Reversed DAPI image of A.

C: *hTERT* (red) co-hybridisation with 5ptel probe 114j18 (green, Cytocell). 5qtel probe was also hybridised but the signal was weak and not apparent in this image.

D: Reversed DAPI image of C.

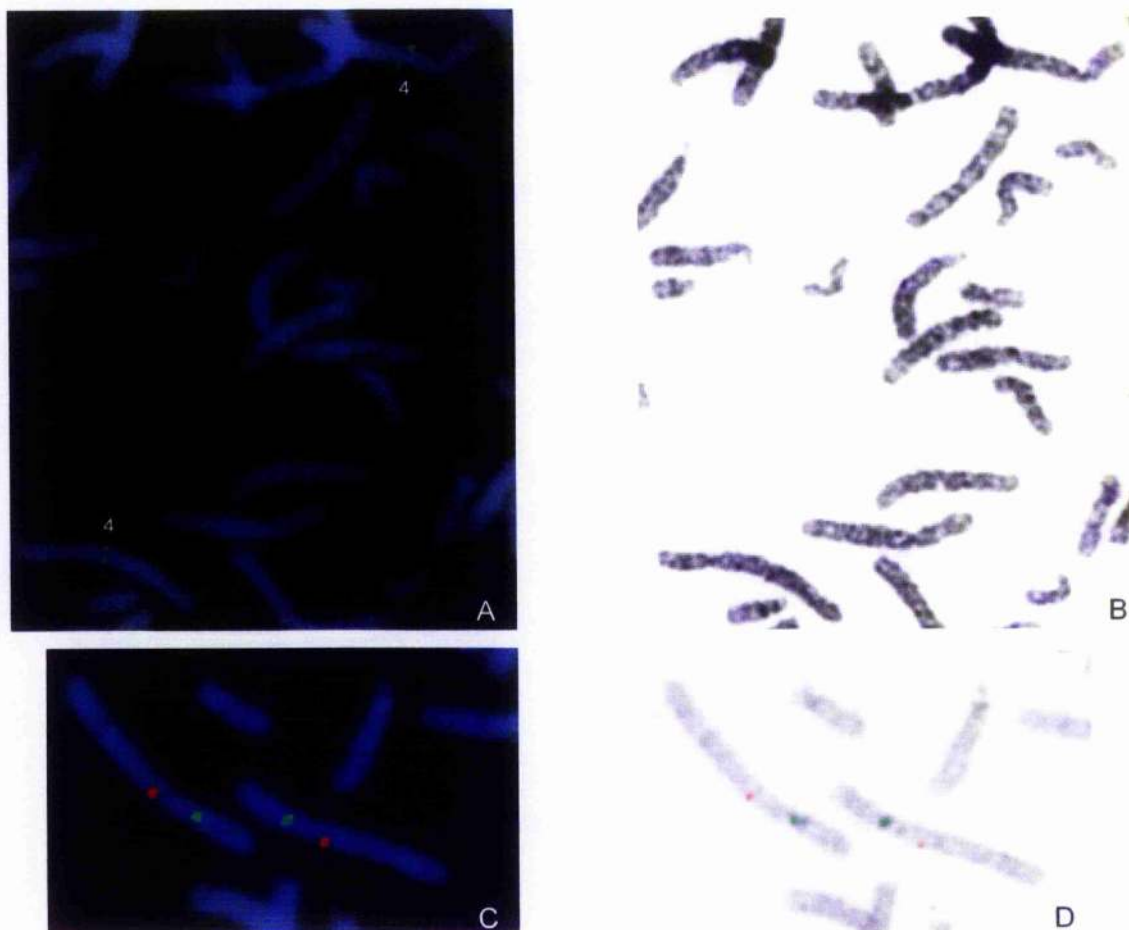


Fig.3-4 Localisation of putative *SCD2* to 4q21.2~q21.3.

A: *SCD2* cosmid 17.

B: Reversed DAPI image of A.

C: *SCD2* cosmid 9 (red) co-hybridisation with chromosome 4 alpha satellite probe (green, Oncor).

D: Reversed DAPI image of C with *SCD2* (red) and chromosome 4 alpha satellite probe signal (green) superimposed.

3.1.4 Stearoyl coenzyme A desaturase 2 (SCD2)

Prior to this mapping experiment rodent studies had identified two *SCD* genes with tissue-specific expression patterns. Human *SCD1* had been completely characterised (MIM 604031) and the gene localised to chromosome 10, whereas *SCD2* had not been widely investigated in humans and its existence was even in doubt (Zhang et al 1999).

As detailed in Appendix I, while attempting to isolate human *SCD2* our collaborators obtained four cosmid clones (9, 17, 22 and 30) with positive hybridisation to a putative *SCD2* sequence. These clones, at concentration 0.18ug/ul, were then supplied for regional localisation by FISH.

The four clones were biotinylated and 9 and 17 hybridised, using a concentration of 10ng/ul, with 1ug/ul total human DNA as competitor. Post-ISH washing was in 0.4xSSC at 72°C for 2 minutes and two layers of avidin FITC were used in detection.

Ten metaphase chromosome spreads were analysed from both the clone 9 and 17 hybridisations. In each cell, signal was seen only on both homologues of a B group chromosome, tentatively identified as chromosome 4 (Fig.3-4).

To verify their chromosome 4 localisation, cosmids 9, 22 and 30 were then hybridised to normal metaphase spreads in combination with 0.5ul DIG-labelled chromosome 4 alpha satellite probe (Oncor) per 10ul of probe mix. Ten metaphase spreads were analysed for each FISH, confirming that the cosmid sequences all mapped to the boundary of chromosome bands 4q21.2 and q21.3.

3.1.5 Chromosome Breakpoint Definition Using Mapped FISH Probes

3.1.5.1 Mapping and Ordering of Chromosome 21-Specific Probes for Breakpoint Definition in a Case of Partial Trisomy 21

Patient DP

The patient was a two day old infant (born at 36+1/40) referred as 'query Down syndrome' with intrauterine growth retardation below the 3rd centile and dysmorphic features including bilateral single palmar crease, microphthalmia and increased distance between 1st and 2nd toes. The infant subsequently died aged 5 days.

Chromosome analysis revealed the presence of a derivative chromosome 12 with an apparent inverted and duplicated segment of chromosome 21 on the short arm:

45,XY,der(12)(12qter→12p13.3::21q22.3→21q11.2::21q11.2→21qter),-21*de novo*

Diagnostic FISH studies confirmed the chromosome 12 breakpoint as 12p13.3 (by chromosome paint end points) and that the 21 material had both 21q22-specific sequence (YAC 831B9, Morris et al 1999) and 21qtel sequence (D21S1575) at its distal and proximal ends but no 13/21 alpha satellite signal:

ish der(12)t(12;21)(wcp21+,D21S1575++,831B9++,D13Z1/D21Z1-,wcp12+).

Blood had been taken from the patient's 27 year-old mother at 16 weeks gestation and serum levels of chorionic gonadotrophin (hCG) and alpha-fetoprotein (AFP) were within normal ranges despite foetal trisomy of almost all of the long arm of chromosome 21. This prompted further definition of the region of 21q monosomic in this patient as part of a collaborative study aimed at the identification of a candidate region involved in the regulation of these serum protein levels in pregnancy.

Test Material

Karyotypically normal metaphase spreads were used for band localisation and preliminary probe ordering before ISH to patient chromosomes. Metaphase spreads were available from patient blood. Gonad had also been cultured for diagnostic purposes.

Probes

Initially three probes which had previously been mapped to 21q11.1-q21 (more precise band localisations were not available) were selected and obtained from the Max-Planck Institute for Molecular Genetics repository database (MPI, Berlin). These particular YACs (949b09, 937e12, 925h10) were chosen, on the basis of FLpter and cMpter values, to span this region and therefore, hopefully, the breakpoint on the duplicated chromosome 21 component of the der(12) in patient DP.

Following initial mapping with these probes which identified that the breakpoint lay between YAC sequences 937e12 and YAC 949b09 (as explained below), further clones believed to lie within this interval were requested. Three, which had not previously been mapped by ISH, were supplied (YACs 858e10, 856h04, 759d03).

YAC inserts were amplified by DOP-PCR essentially according to the MPI method and labelled, following yield determination (Table 3-3), by nick translation with biotin and/or DIG.

PROBE	DILUTION	ABSORBANCE 260nm	CONCENTRATION
925h10	1/200	0.034	0.34ug/ul
949b09	1/200	0.058	0.58ug/ul
937e12	1/200	0.051	0.51ug/ul
759d03	1/200	0.035	0.35ug/ul
858e10	1/200	0.038	0.38ug/ul
856h04	1/200	0.026	0.26ug/ul

Table 3-3 DNA yield following DOP-PCR amplification of chromosome 21-specific YACs.

Hybridisation Results

Preliminary hybridisations were performed to discern optimal signal amplification and probe and competitor DNA concentrations for biotinylated probes. These conditions were re-assessed and if necessary adjusted for DIG label and dual hybridisation (Table 3-4).

Single and dual colour ISH to normal metaphase spreads (~550 band chromosomes) provided refined band localisation and, where possible, clarified or confirmed the order of these six sequences. Results (Fig.3-5, Tables 3-5, 3-6), suggested the order:

21cen-949b09or858e10-759d03-856h4or937e12-925h10-21qter

Hybridisation to patient DP showed YAC 925h10 and 937e12 sequences were present centrally on the chromosome 21 region of the derivative chromosome 12 as two copies and an indeterminate copy number respectively (Fig.3-6). YAC 949b09 sequence was absent, indicating, as 925h10 and 937e12 order had been determined, that the breakpoint on at least one chromosome 21 component lay between 937e12 distally and 949b09.

Though of adequate quality for probe ordering, YACs 759d03 and 858e10 produced less consistent ISH signals than the others. When the stored gonad cultures from patient DP failed to grow on reconstitution it was therefore decided that, to conserve the small amount of patient material remaining, alternative clones with higher hybridisation efficiency should be sourced for any further mapping of the breakpoint.

Six proximal 21q21.1 clones (RP11 141D02, 184E23, 65E08, 280N08, 268F23 and RP1 152M24) were therefore later selected using the Ensembl Genome Browser (Birney et al 2004, Fig.4-5) and ordered from the Human BAC Resource, The Sanger Centre, Cambridge. The cultures received were grown in broth containing chloramphenicol, or in the case of RP1 152M24, kanamycin, and probes extracted using a 'miniprep' technique. 5ul of BAC/PAC DNA was biotinylated by nick translation. ISH using an estimated 6ng of probe/ul with 1ug/ul genomic competitor DNA confirmed that all did map to proximal 21q. The two most proximal sequences according to Ensembl, 268F23 (16.03Mb) and 280N02 (16.1Mb), were hybridised to patient metaphases, the latter with 2ug/ul competitor DNA. Both sequences were present on the der(12) chromosome, with indeterminate copy number. This refined at least one breakpoint on the duplicated chromosome 21 region of the derivative 12 to between 949b09 proximally and 268F23 distally (Table 3-7).

Use of 21-Specific YACs to Aid Definition of an Abnormal Chromosome 21 in Patient LF
YACs 949b09 and 937e12 were also hybridised to a chromosome preparation from LF (Appendix II), a patient with karyotype 46,XX,?del(21)(p). Although the ?del(21)(p) and normal chromosome 21 could not be differentiated in the poor quality metaphase preparations available, all eight cells examined showed no deletion of these sequences on either chromosome. The more proximal location of 949b09 was confirmed (Fig 3-6).

PROBE NAME	PROBE ng/ul	COMPETITOR DNA	FLUOR LAYERS	CHROMOSOMES 21 WITH SIGNAL	HYBRIDISATION QUALITY
925h10 BIO	5	1ul Cot1	2	100% (20/20)	signal large, some b/g
	10	2ul Cot1	2	IA	as 5ng/ul
	15	2ul Cot1	2	IA	as 5ng/ul
	20	2ul Cot1	2	IA	as 5ng/ul
925h10 DIG	6 DH	2ul Cot1	2	100% (11/11)	signal strong, no b/g
949b09 BIO	5	1.5ul Cot1	2	90% (36/40)	some b/g, little cross hybridisation, easier to analyse without imaging than single layer
	5	1.5ul Cot1	1	IA	no b/g, see above
	6 DH	2ul Cot1	2	100% (12/12)	signal strong, no b/g
949b09 DIG	6 DH	2ul Cot1	2	96.6% (29/30)	signal large
	10	2ul Cot1	2	IA	signal excessively large
937e12 BIO	5	1.5ul Cot1	2	50% (12/24)	some cross hybridisation, b/g, easier to analyse without imaging than single layer
	5	1.5ul Cot1	1	IA	see above
	8 DH	2ul Cot1	2	80% (24/30)	slight b/g
	10 DH	2ul Cot1	2	100% (11/11)	some b/g, signal consistent
759d03 BIO	10 DH	2ul Cot1	2	68.8% (11/16)	some b/g
856h04 BIO	10 DH	2ul Cot1	2	90% (9/10)	some b/g
856h04 DIG	10 DH	2ul Cot1	2	89.5% (17/19)	slightly weak but acceptable
858e10 BIO	10 DH	2ul Cot1	2	16.7% (5/30)	weak, b/g
	16	1ul genomic	2	5% (1/20)	weak, very infrequent signal
	16 DH	2ul Cot1	2	14.7% (5/34)	very weak

Table 3-4 Hybridisation conditions assessed for chromosome 21 YACs.
b/g: background signal; DH: dual hybridisation; IA: incomplete analysis

YAC	FLpter (MPI)	cMpter (MPI)	CHROMOSOME 21 BAND	
			MPI	THIS STUDY
949b09	0.15-0.2	0	q11.1~q21	q11.2
858e10	0.15-0.2	unknown	unknown	q11.2
759d03	unknown	unknown	unknown	q11.2
856h04	0.15-0.2	9	unknown	q11.2~q21.1
937e12	0.15-0.2	9/13	q11.1~q21	q11.2~q21.1
925h10	0.30-0.37	21	q11.1~q21	q21.1~q21.2

Table 3-5 Metaphase mapping to determine localisation of chromosome 21 YACs.
949b09, 937e12 and 925h10 were hybridised singly. Dual FISH (with 949b09) was used for 858e10, 856h04 and 759d03. MPI map data, if available, was obtained from the MPI website.

POSITION OF:	RELATIVE TO:	NO. OF CHROMOSOMES			TOTAL NUMBER OF CHROMOSOMES SCORED
		proximal	even	distal	
949b09	858e10	0	10	0	10
949b09	759d03	2	9	0	11
949b09	856h04	8	1	0	9
949b09	937e12	20	4	0	24
856h04	937e12	6	9	2	17
949b09	925h10	6	0	0	6
937e12	925h10	11	0	0	11

Table 3-6 Dual colour metaphase ordering of chromosome 21 YACs.

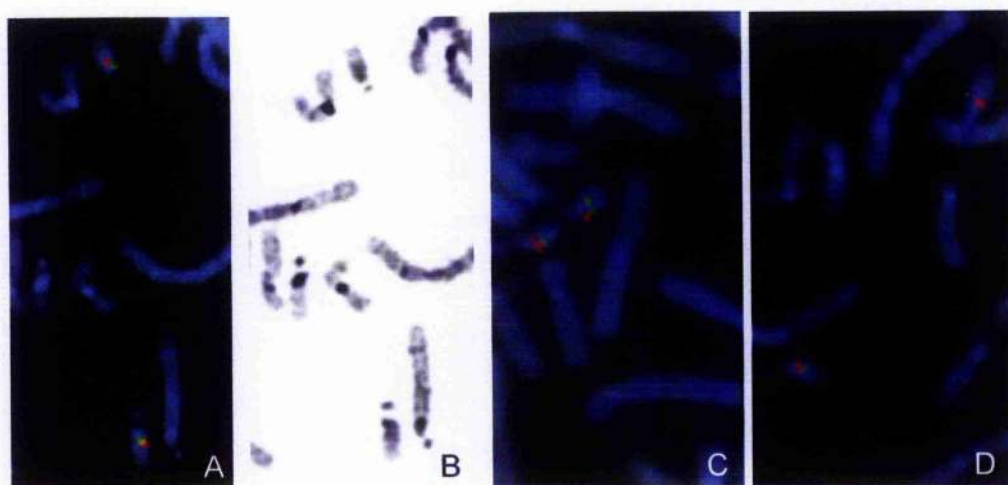


Fig.3-5 Mapping and ordering of chromosome 21-specific YACs on normal metaphases.

A: 949b09 (green, proximal) and 937e12 (red).

B: Reverse DAPI image of A.

C: 937e12 (red, proximal) and 925h10 (green).

D: 949b09 (red, proximal) and 856h04 (green).

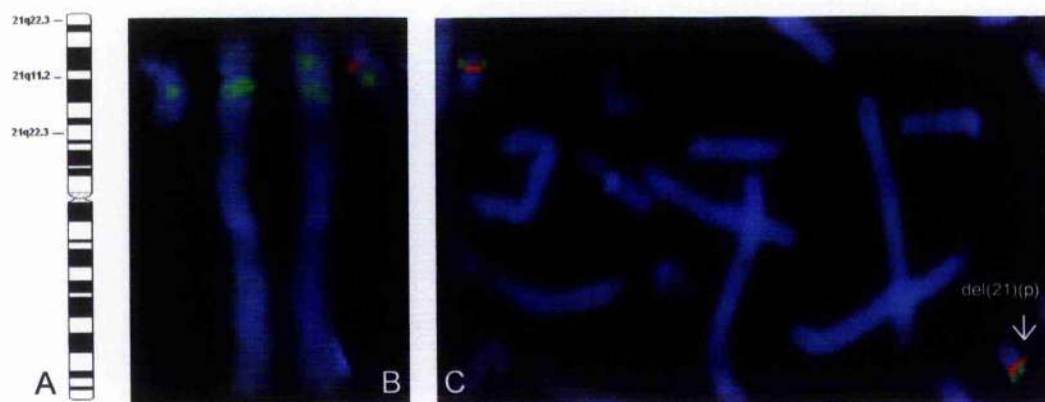


Fig.3-6 Hybridisation of chromosome 21-specific YACs to patient metaphases.

A: Ideogram of der(12)(12qter→12p13.3::21q22.3→21q11.2::21q11.2→21qter) in DP.

B: left - 937e12 on normal 21 and on der(12).

right - 949b09 (red) and 925h10 on normal 21 and on der(12). Note 949b09 signal is not present on the chromosome 21 component of the der(12) chromosome.

C: 949b09 (green) and 937e12 ISH to a metaphase from patient LF.

PROBE	STS MARKER	FISH STUDY
949b09	D21S1911	DELETED
RP11 268F23	D21S189/1830	NOT DELETED
RP11 280N08	D21S189/1830/1432	NOT DELETED
937e12	D21S1256/1699	NOT DELETED
925h10	D21S1257	NOT DELETED

Table 3-7 Breakpoint mapping on der(12)t(12;21) in patient DP.

STS:sequence tagged site associated with probe sequence
(source MPI or Ensembl Genome Browser).

3.1.5.2 Mapping and Ordering of Chromosome 8-Specific Probes for Orientation of Duplicated Region and Breakpoint Definition in a Case of 8p Duplication

Patient RM

The patient was referred, when three years old, with mild global delay, language and social problems and mild dysmorphic features (detailed in Appendix II).

Chromosome analysis revealed the presence of an abnormal chromosome 8 with an apparently duplicated region on the short arm, karyotype: 46,XX,dup(8)(p11.2p21.3).

Preliminary FISH investigations showed normal signal patterns with chromosome 8 paint and telomere probes. As direct duplications of proximal 8p are rare, breakpoint and orientation investigations were initiated with BAC probes from this region.

Probes

Four clones, RP11 350N15, RP11 564K10, RP11 527N22 and RP11 23D17, estimated to lie within the duplicated region (and hence be useful for orientation studies) were selected using Ensembl and ordered from the Human BAC Resource at the Sanger Centre. A clone labelled 23D7 was sent in place of 23D17.

The bacterial stab cultures were first plated on chloramphenicol agar. Selected colonies were cultured and extracted using the 'miniprep' technique. Gel electrophoresis was carried out to ascertain that high molecular weight (BAC) DNA had been obtained (Fig.3-7).

5ul of BAC DNA was either biotinylated (RP11 350N15 and 564K10) or labelled with DIG (RP11 527N22 and 23D7) using nick translation. Dual hybridisation using 10ng/ul of each probe and 1.5ug/ul human genomic competitor DNA confirmed that all except 23D7 did map to 8p, in order 8cen-350N15-527N22-564K10-8pter, and were included in the duplicated region. 23D7 mapped to 2p16.

ISH of DIG-labelled RP11 527N22 (FITC detection) with biotinylated RP11 564K10 (Texas Red detection) produced an alternating green/red signal pattern (Fig.3-7). This confirmed that the duplication was not inverted.

Further clones, generously provided by Dr J Fantes (MRC, Western General Hospital, Edinburgh), were selected from Ensembl to further define the duplicated area. These were biotinylated and individually hybridised to patient metaphases at an estimated 6ng/ul with 1ug/ul human genomic competitor DNA (initial experiments with 10ng/ul probe produced excessive background).

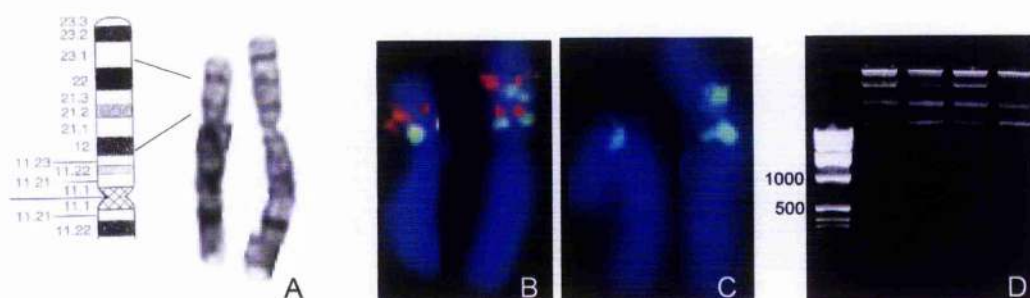


Fig.3-7 Breakpoint mapping of 8p duplication in patient RM

- A. G banded image (normal chromosome 8 and ideogram of 8p on the left).
 B. FISH with BACs RP11 527N22 (FITC label) and 564K10 (Texas red) confirms that the duplication is direct and involves both 8p12 BAC sequences.
 C. FISH with 8 alpha satellite centromeric probe (8p11.1) confirms that these centromeric sequences are included in the duplication.
 D. Agarose gel electrophoresis of extracted BAC DNA.
 Lanes from left: 1kb size ladder; 23D7; RP11 527N22; 564K10; 350N15.
 Only high molecular weight DNA is apparent.

CLONE	ENSEMBL MAP LOCATION		FISH RESULT
RP11 369E15	8p21.3	20.67Mb	not dup
RP11 177H13	8p21.2	23.07Mb	not dup
RP11 395I14	8p21.2	25.12Mb	not dup
RP11 14I17	8p21.2	26.17Mb	not dup
RP11 138J2	8p21.2	27.24Mb	not dup
RP11 356F24	8p21.1	28.39Mb	dup
RP11 263C6	8p12	28.89Mb	dup
RP11 564K10	8p12	29.19Mb	dup
RP11 527N22	8p12	37.12Mb	dup
RP11 350N15	8p12	38.22Mb	dup
RP11 44K6	8p11.21	39.73Mb	dup
RP11 51K12	8p11.21	40.39Mb	dup
RP1 198M21	8p11.21	41.41Mb	dup
RP11 231D20	8p11.21	42.04Mb	dup
RP11 137L15	8q11.21	48.57Mb	not dup

Table 3-8 Hybridisation of chromosome 8-specific clones to a duplication of 8p in patient RM. Clones used for initial, dual colour, duplication orientation are shown in bold type.

Analysis of at least three metaphases from each hybridisation (the results of which are recorded in Table 3-8) demonstrated that the chromosome 8 breakpoints were actually at 8p11.1~p11.2 and 8p21.1~21.2. Clone RP11 138J2, excluded from the duplicated region, lies 0.03Mb from the 8p21.1-21.2 junction. Clone 137L15, which lies < 0.6Mb from the centromere in band 8q11.21, was also not included in the duplication, but ISH to 8 alpha satellite centromeric probe (Qbiogene, Cambridge) showed 8 alpha satellite signal had been duplicated, further refining the proximal breakpoint:

46,XX,dup(8)(p11.2p21.3).ish dup(8)(p11.1p21.1~p21.2)(356F24++,263C6++,
564K10++,527N22++,350N15++,44K6++,51K12++,RP1 98M21++,231D20++,D8Z1++)

3.2. Detection of Cryptic Chromosomal Abnormality

3.2.1 DG/VCF Syndrome Microdeletion Testing

The objectives of this study were primarily to establish a diagnostic test and, in the earlier stages, collect some basic information on deletion size by use of a dual locus probe known to be deleted at only one locus in some individuals (Lindsay et al 1995).

When the study began, the DG/VCFs critical region was only loosely defined and no commercial probe was available, so it was necessary to culture and label probe 'in house'. As it progressed probe selection altered because new clones, considered possibly to have improved ISH efficiency, specificity, or diagnostic utility, were recommended by the probe developers. Probe and patient sample details are in Appendices I and II.

DG/VCF Syndrome Probes Used

Five different DG/VCFs region cosmids were made available over an eighteen month period. These were plated out on either ampicillin (sc4.1) or kanamycin-containing agar. Five colonies were picked from each plate, starter cultures incubated and glycerol stocks prepared from these for storage at -20°C. One or two of these starter cultures or glycerol stocks from each of the cultures were used to inoculate 100ml LB broth for further culture and subsequent cosmid isolation using the Circleprep kit (Bio 101). The DNA yield was typically 0.21-0.44 ug/ul (21-44ug).

The earliest investigations utilised cosmids sc4.1 (D22S134) and sc11.1, which detected two loci, sc11.1a and sc11.1b, at opposite ends of the commonly deleted 2-3Mb region. Two patients, one deleted, were tested with only one of these probes due to shortage of patient material. Two relatives of suspected microdeletion cases were tested only with sc11.1, one because of shortage of material and the other because testing was

discontinued when deletion was not detected in his son. Investigation of two relatives of recognised deletion cases, who had an untested or deletion-negative intervening relative, was restricted to sc11.1 testing because this probe was informative in the probands. Combinations of sc11.1 and/or sc4.1 with E0472 (D22S697), D0832 (D22S502) and eventually H1012 were subsequently used. By the end of the study, most cases were being tested only with H1012, as this contained the *TUPLE 1* sequence which was considered to be a strong candidate gene for DG/VCFS. Other probes were then only employed if a negative result was obtained in conjunction with strong clinical suspicion of 22q11.2 deletion. Cosmid H1012 shares some sequence with commercial *TUPLE 1* probes. The order of these sequences is shown in Figs.1-11 and 3-8. When testing relatives of deleted individuals the probe (or one of the probes) employed had been shown to disclose deletion in the proband.

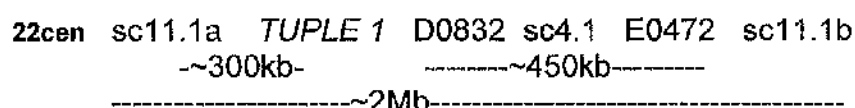


Fig.3-8 Order of probes used for DG/VCFS microdeletion testing. Determined by pulsed field gel or interphase FISH measurements (Lindsay et al 1993, Halford et al 1993a).

Hybridisation Strategies and Conditions

Following preliminary ISH experiments, biotin labelling and amplified avidin-FITC detection was used for all probes as it proved difficult to obtain signal of adequate strength from DIG labelling without unacceptably high background, despite altering probe concentration and wash stringency. A working probe concentration of 10ng/ul was established for sc11.1, sc4.1 and D0832, and 15-20ng/ul for E0472. Total human genomic DNA (1ug/ul) was used as competitor. A rapid 'combined' detection method which (by providing a mix of appropriate detection reagents) produced amplification but required only a single detection step, was tried with sc11.1 and E0472. This approach was not adopted as it generally required digital imaging, rather than conventional microscopy, for satisfactory visualisation of signals on both homologues in most cells. In an attempt at dual colour FISH which avoided the problems associated with DIG label while reducing detection time, sc4.1 and sc11.1 were directly labelled with fluorogreen and fluorored (Amersham), but the signal produced was too weak for diagnostic purposes. To provide a control signal in co-hybridisation with these directly labelled DG/VCFS probes, chromosome 22 alpha satellite centromeric probe (22/1:2.1, ATCC 61578) was cultured, extracted, directly-labelled (fluorogreen or fluorored) and used at 5-10ng/ul. Again the direct signal obtained was weak, despite modifying the ISH and wash conditions, though biotinylated, indirectly detected 22 centromere probe signal was

strong. FISH with another similarly extracted and directly labelled sequence, chromosome 18 alpha satellite probe (L184, ATCC 61394), did produce satisfactory signal, but optimisation of 22 alpha satellite co-hybridisation was not pursued as the introduction of digital imaging allowed chromosome identification by reverse DAPI banding, making control signals unnecessary.

Analysis

Initially ten, then, with experience, five cells were scored for the presence of probe signal on one or both chromosomes 22 (Fig.3-9). If available, more metaphase spreads, up to thirty if necessary, were scored where deletion was suspected.

Patients Tested and Outcome of Testing

Of the 244 individuals tested with in-house probe, 161 were referred because they exhibited phenotypic features of DG/VCFS (Table 3-9). Deletion was found in 34 (21.1%), including the three patients with unusual signal patterns listed in Table 3-13 and discussed below. Only one of these 34 deletions was visible cytogenetically.

Eighty three individuals were referred for testing primarily because of their relationship to affected individuals, rather than possession of a DG/VCFS phenotype. These included:

- Forty six parents, from twenty four two-generation and three, three-generation families, and one child of a deleted individual. Five parents were deleted (Table 3-10). One exhibited VCFS but the other deleted parents had not been considered to have a 'syndromic' phenotype. One parent had submucous cleft palate and transient hypocalcaemia at birth, one had mild learning difficulty, one had mild learning difficulty and a VSD and the other exhibited a degree of brachycephaly.
- Six relatives of deleted individuals whose intervening relative(s) were of unknown or negative deletion status. Though one patient, whose sister and nephew were deleted, was described as "query VCFS" no microdeletions were found in this group (Table 3-11).
- Thirty parents or other relatives of individuals who either had suspected microdeletion but subsequently tested negative or who were not available for testing. Again, no microdeletions were found (Table 3-12).

PROBES USED	CASES STUDIED	CASES DELETED
sc11.1/sc4.1	15	4
sc4.1	1	1
sc11.1	1	0
sc11.1/sc4.1/E0472	7	2
sc11.1/sc4.1/E0472/H1012	2	1
sc11.1/E0472	45	6
sc11.1/E0472/H1012	3	1
sc11.1/sc4.1/E0472/D0832/H1012	1	0
sc11.1/H1012	12	5
H1012	74	14

Table 3-9 Breakdown of 22q11 probe combinations used to test 161 affected individuals.

PROBES USED	CASES STUDIED	CASES DELETED
sc11.1/sc4.1	9	2
sc11.1/sc4.1/E0472	1	1
sc11.1/E0472	10 (<i>child in this group</i>)	1
sc11.1/H1012	2	0
H1012	25	1

Table 3-10 Probe combinations used to test 46 parents and 1 child of deleted individuals.

PROBES USED	CASES STUDIED	CASES DELETED
sc11.1/sc4.1	1	0
sc11.1	2	0
sc11.1/H1012	1	0
H1012	2	0

Table 3-11 Probe combinations used to test 6 relatives of deleted individuals. The intervening relative(s) were either not tested (4 cases) or subsequently shown not to be deleted (2 cases).

PROBES USED	CASES STUDIED	CASES DELETED
sc11.1/sc4.1	2	0
sc11.1/sc4.1/E0472	1	0
sc11.1/E0472	9	0
sc11.1	2	0
sc11.1/E0472/H1012	2	0
E0472/H1012	2	0
sc11.1/H1012	1	0
H1012	11	0

Table 3-12 Probe combinations used to test 30 relatives of individuals who either had suspected microdeletion but subsequently tested negative or who were not available for testing.

Possible Atypical DG/VCFS Region Deletions

Not all DG/VCFS cases tested produced unequivocal probe ISH results (Table 3-13).

Three of the 34 patients in whom hemizygosity was clearly demonstrated (JF, KA, HG) showed deletion of H1012 sequence but sc11.1 signal on both chromosomes 22 in 33-60% of metaphase spreads. In all other sc11.1 hybridisations to patient material with deletion at another DG/VCFS probe locus no cells examined exhibited sc11.1 signal on

both chromosomes 22. The presence of a second sc11.1 signal, at reduced frequency, suggested that these deletions did not include one sc11.1 repeat (a or b).

Unfortunately, hybridisation to other probes either failed or gave ambiguous results. The low number of cells with signal on both chromosomes 22 with probe E0472 (in patients JF and KA) could conceivably represent background. While generally of acceptable quality, variable hybridisation efficiencies and background levels could occasionally be associated with these probes, particularly on very poor chromosome preparations. However, one alternative explanation for the apparent incomplete deletion of E0472 in cases JF and KA is that E0472 sequence, like sc11.1b, appears to lie within the markers flanking the distal breakpoint in smaller deletions (Morrow et al 1995, Carlson et al 1997).

As no patient material remained these investigations could not be repeated to confirm results or exclude the possibility that poor quality hybridisations were responsible for the ambiguous signal patterns with probe E0472.

PATIENT	SIGNAL ON BOTH CHROMOSOMES 22		
	sc11.1	H1012	E0472
JF	6/10	0/17	5/20 <i>2nd signal pale</i>
KA	6/18	0/10	1/10
HG	5/10	0/19	not tested

Table 3-13 Apparently atypical deletions in three DG/VCFS patients.

Inheritance of 22q11.2 Deletion

Testing was able to be performed on one or both parents of 27 of the deleted patients, three of whom were themselves parents of deleted children. Whether a deletion was inherited could be determined in 21 of these 27 patients either because they had both parents tested or because the single parent who was tested exhibited deletion. Five deletions were detected in this group of 21, therefore 23.8% of deletions could be confirmed as inherited. Four of the five inherited deletions were maternal in origin.

Microdeletion Testing in Patients with Tetralogy of Fallot

As hemizyosity at 22q11.2 had been reported in patients with non-syndromic conotruncal defects (Wilson et al 1992a), a series of 24 patients diagnosed with TOF were examined for microdeletion at the DG/VCFS locus (Table 3-14). Deletion at 22q11.2 was detected in four patients. Whether a deletion was inherited could be determined in three of these cases, and one maternal deletion was demonstrated. The deleted parent was subsequently described as "possibly having some facial features of VCFS" but had no cardiac defect or any other apparent abnormality.

PROBES USED	CASES STUDIED	CASES DELETED
sc11.1/sc4.1/E0472/D0832	3	2
sc11.1/E0472	19	0
sc11.1/H1012	1	1
sc11.1	1	1

Table 3-14 Probe combinations used to test 24 patients with TOF.

The results from this series were combined with those from individuals with TOF in the main DG/VCFS series. In total, seven deletions were disclosed in thirty three individuals with TOF. Three of the seven patients had, in the opinion of a clinical geneticist, isolated TOF with no other suggestive clinical features (Trainer et al 1996).

22q11 Deletion Diagnosis With Commercial Probes

Studies with commercial probes (Appendix I), principally for the *TUPLE 1* region, have continued as part of the routine diagnostic service. *TUPLE 1* probe rather than N25 was chosen both for continuity, as the *TUPLE 1* sequence was present in the 'home-grown' H1012 probe, and because *TUPLE 1* was a candidate gene.

In the course of providing this service patient KC, who had been shown by another cytogenetics laboratory not to be deleted at the N25 (Oncor) locus, was tested with *TUPLE 1* probe (Vysis) because of a strongly suggestive phenotype including VSD, interrupted aortic arch, absent thymus and hypocalcaemia. Deletion was detected. To determine the extent of the deletion, 22q11 microsatellite analysis was performed by the Molecular Genetics Service, Western General Hospital, Edinburgh and showed homozygosity for all markers tested telomeric to D22S1638. This was strongly suggestive of a typical distal deletion junction at or near D22S938. Heterozygosity for marker D22S1638 confirmed the proximal breakpoint was unusual. However, as this marker proximally flanks N25 and the closest telomeric marker (D22S1648) distally flanks *TUPLE 1*, this test could not, unlike FISH, discern that *TUPLE 1* was deleted but N25 was not.

As a result of finding this variant deletion most 'query 22q11 deletion' referrals to this department are now tested with both N25 and *TUPLE 1* probe. In over 100 cases tested with both probes to date all are either undeleted or deleted for both sequences.

By the end of 2003, 855 patients - of which 100 had a deletion - and 206 relatives had been tested (excluding the TOF series). Inheritance could be confirmed or excluded for 59 of these deletions, and 11 (18.6%) were shown to be inherited, 9 of them (81.8%) from the mother. Thirteen (13%) of the deletions were cytogenetically visible. Prenatal diagnosis was performed on four pregnancies of deleted individuals (deletion was detected in one foetus) and on two pregnancies with cardiac defects (not deleted).

Other Cytogenetic Abnormalities Found in Patients Referred for '22q Deletion Testing'
 By January 2004 thirteen cytogenetic abnormalities, some of which may not be pathological, had been found in patients referred for testing with DG/VCFS probe (Table 3-15).

CASE NUMBER	CHROMOSOME ABNORMALITY
202847	46,XY,inv(10)(q11.2q21.2)
203408	46,XX,tra(10)(q23.3)
963498	46,XX,v(20)(cen)
963751	46,XY,t(1;3)(q25;q27)
970814	46,XX,dup(1)(q42;q43)
970984	46,XY,inv(9)(p11q13)
971481	47,XYY
975771	46,XY,add(21)(q22.3)
993196	46,XY,der(3)t(3;5)(p26;p13)
995648	46,XY,inv(12)(q22q24.22)
230161	47,XY+13 [7]/46,XY[43]
231503	46,X,ix(q10)
236483	46,XY,t(2;3)(p15;q21)

Table 3-15 Cytogenetically visible chromosome abnormalities, other than 22q11 deletion, found in individuals referred to the Duncan Guthrie Institute for DG/VCFS FISH testing.

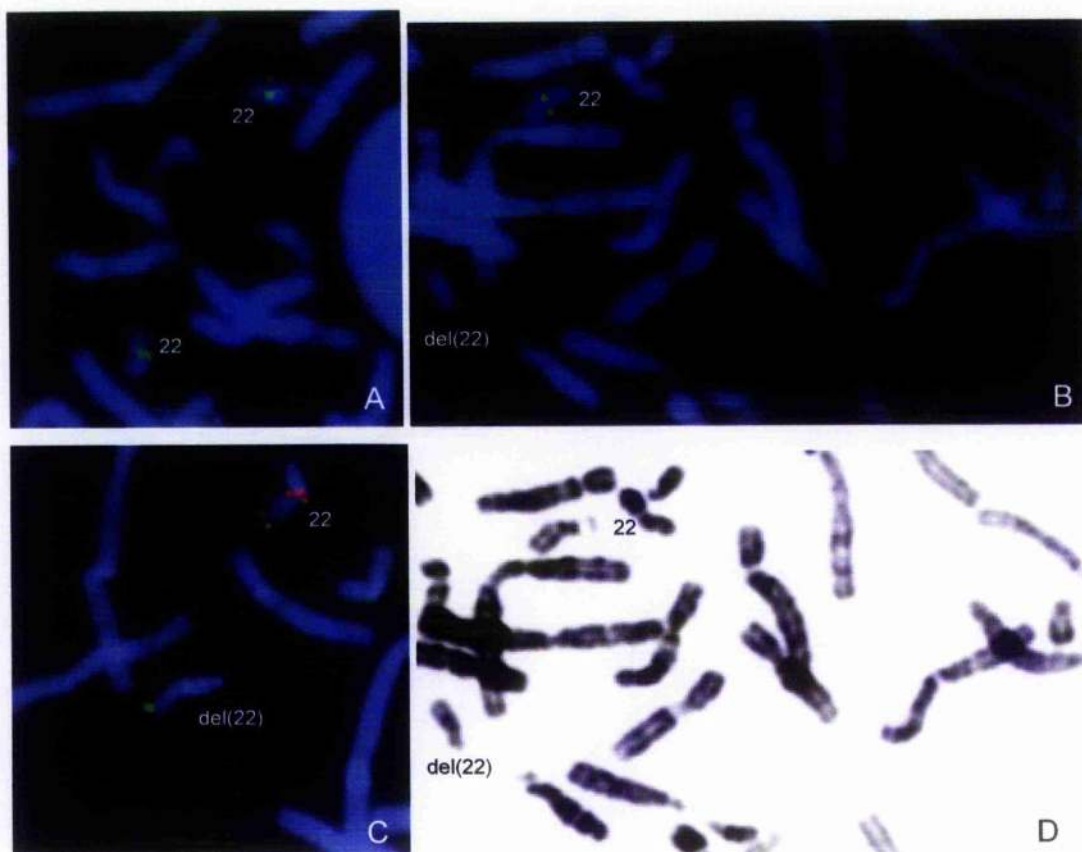


Fig.3-9 FISH with DG/VCFS region probes.

A: Cosmid sc11.1 hybridised to a metaphase from an individual with no 22q11 deletion. Signals are present on both chromosomes 22.

B: Cosmid sc4.1 hybridised to a metaphase from a patient with a deletion at 22q11. Signals present on only one chromosome 22. The presence of four other G group chromosomes in this 46XY cell was confirmed by reverse DAPI banding (see image D below).

C: Commercial *TUPLE 1* probe (Vysis) hybridised to a metaphase from a patient with a deletion at 22q11. *TUPLE 1* signal (red) is present on only one chromosome 22. Green control signal (ARSA sequence, 22q13.3) identifies both chromosomes 22.

D: Reverse DAPI banding of image B, showing presence of both chromosomes 22.

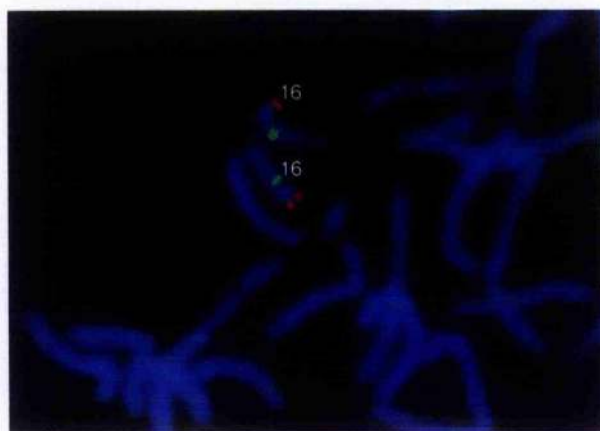


Fig.3-10 Hybridisation to biotinylated RT1 cosmid (16p13.3) and DIG-labelled chromosome 16 alpha satellite probe.

Neither chromosome 16 shows deletion of RT1 sequence (red signal).

3.2.2 Detection in a Large Pedigree of a Cryptic Chromosomal Rearrangement Involving the Wolf-Hirschhorn Syndrome Locus

ISH with mainly non-commercial probes on metaphase preparations from blood and/or lymphoblastoid cell lines was used to demonstrate the segregation of a submicroscopic reciprocal translocation between chromosomes 4 and 11 in an extensive family (Fig.3-11).

Patients

The proband, V-11 and her second cousins once removed (IV-8 and IV-10) displayed a collection of severe congenital defects (detailed in Appendix II). Unusual prominence of the glabella and nasal root in the proband led to a tentative clinical suspicion of WHS. However high resolution study of banded chromosome preparations in addition to flow cytometry karyotyping in this department revealed no detectable abnormality of 4p. This prompted molecular cytogenetic studies.

Probes and Hybridisation Strategies

Plasmid pK082 was cultured with ampicillin selection, extracted (Circleprep, yield 100ul, 0.35ug/ul), and biotinylated, generally by nick translation, for use as a region 4p16.3 probe. For early investigations on V-11, IV-11 and V-12, all poor cytogenetic preparations, marginally better results were obtained using pK082 biotin-labelled by random priming (Feinberg and Vogelstein 1983) with a commercial kit (Boehringer Mannheim Random Primed DNA Labelling Kit 1004760).

Initially region 11p15.5-specific plasmid phins310, then phins311, were similarly prepared (yield 100ul, 0.31ug/ul and 0.11ug/ul respectively) and biotinylated by nick translation. Following optimisation experiments, early pK082 hybridisations (to V-11, IV-11, IV-12, IV-16, IV-17) employed 1-2ng/ul probe chromogenically detected (Garson et al 1987). Plasmid phins310 hybridisations, which were performed only on the proband, her parents and two affected second cousins once removed (V-11, IV-11, IV-12, IV-8, IV-10), used 5-10ng/ul biotinylated probe, also detected enzymatically.

Subsequent investigations used 5-10ng/ul pK082 or phins311 for FISH detected with three layers of avidin-FITC following reduced stringency post-ISH washing (two 50% formamide washes at 42°C, each for two minutes). The quality of plasmid phins311 FISH was improved with CISS (0.5ug/ul total human DNA).

Two of three PND investigations employed commercial WHS probe (D4S96) and 4p and 11p telomere sequences (all from Oncor) following ascertainment of their informativeness.

Analysis

In the earliest ISH experiments with the small plasmid probe sequences, signals visualised on post-ISH metaphases were marked on pre-hybridisation, banded, photographs of these metaphases, then on an ideogram, and the chromosomal distribution of signals analysed statistically using the χ^2 test (Fig.3-12). Chromosome identification for signal distribution assessment was subsequently by software enhanced reverse DAPI banding or co-hybridisation with centromeric marker probes.

The proband had signal at 4p16.3 on only one chromosome 4 following ISH to pK082, which confirmed the clinical suspicion of WHS. The same probe produced signal on both chromosomes 4 in the proband's mother but a signal peak on one chromosome 4 and one 11, at 11p15.5, in her father. Confirmation of a familial translocation involving chromosomes 4 and 11 was initially sought with plasmid phins310. This very short sequence (0.8kb) did demonstrate small signal peaks at 4p16.3 and 11p15 in individuals V-11, IV-11, IV-8, and IV-10 but, except in V-11 and IV-8, the peak on 11 was usually not statistically significant ($0.5 > p > 0.1$). The poor hybridisation efficiency of this 11p15.5-specific probe led to its replacement with phins311.

To summarise ensuing experiments (Table 3-16), ISH to pK082 and phins311 revealed cytogenetically cryptic monosomy at distal chromosome 4p and trisomy at distal 11p in the proband and her affected second cousins once removed. Four phenotypically normal individuals in the family, IV-7, IV-11, IV-14 and III-6, were shown to have a t(4;11) translocation in the balanced form (Fig.3-13 A,B) and normal karyotypes were confirmed in seven subjects (including the proband's mother).

To estimate approximately the size of the translocated segments, and to investigate the usefulness of chromosome paints for the demonstration of the translocation in this family, whole chromosome 4 and 11 paints (Cambio, Cambridge) were hybridised to metaphases from a translocation carrier (IV-11). Chromosome 11 material was only just detectable with 11 paint on the der(4) in six of sixteen metaphases, even to an analyst aware of the abnormality, and 4 paint detected translocated 4 material on the der(11) in four of ten metaphases.

Prenatal Analyses

PND was successfully performed for one female carrier (IV-7) of the balanced t(4;11) translocation using FISH on cultured amniocytes. This showed the foetus (V-7) to be a karyotypically normal female: 46,XX,ish 4p16.3(pK082x2),11p15.5(phins311x2)

Two subsequent prenatal FISH investigations were performed for this patient using the WHS probe (D4S96) and telomeric probes for the short arms of chromosome 4 (D4F26)

and 11. The first, on a chorionic villus preparation (V-8), demonstrated partial monosomy 4p and partial trisomy 11p as in the proband (Fig.3-13 C,D):

46,XX.ish der(4)t(4;11)(p16.3;p15.5)mat(D4S96-,D4F26-,11ptel+)

The other, an amniotic fluid sample (V-9), yielded a normal foetal karyotype (Fig.3-13 E,F): 46,XX.ish 4p16.3(D4F26x2,D4S96x2),11p15.5(11ptelx2)

PATIENT	pK082 RESULT*	phins311 RESULT*	KARYOTYPE
V-11	one 4	one 4, both 11	ish der(4)t(4;11)
IV-11	one 4, one 11	one 4, one 11	ish t(4;11)
IV-12	both 4	NOT TESTED	NORMAL
IV-8	one 4	one 4, both 11	ish der(4)t(4;11)
IV-10	one 4	NOT TESTED**	ish der(4)t(4;11)
IV-16	both 4	NOT TESTED**	NORMAL
IV-17	both 4	NOT TESTED**	NORMAL
IV-7	one 4, one 11	one 4, one 11	ish t(4;11)
III-6	one 4, one 11	one 4, one 11	ish t(4;11)
IV-14	one 4, one 11	one 4, one 11	ish t(4;11)
IV-21	both 4	both 11	NORMAL
IV-4	both 4	both 11	NORMAL
IV-1	both 4	both 11	NORMAL
III-10	both 4	both 11	NORMAL

Table 3-16 Results of probe pK082 (4p16.3) and phins311 (11p15.5) hybridisations to chromosomes from members of a family with a cryptic t(4;11) translocation.

*Chromosomes with signal. **Insufficient material.

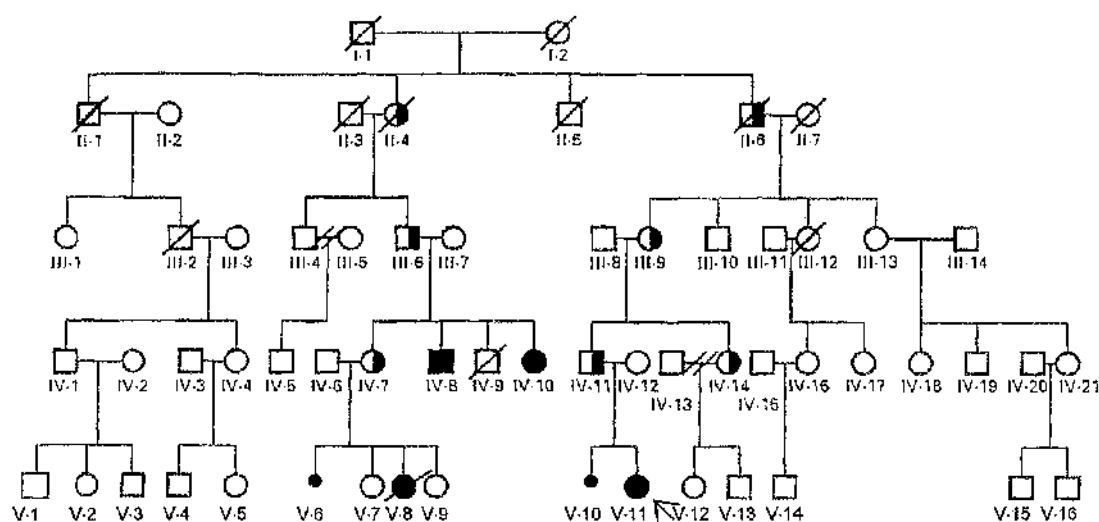


Fig.3-11 Pedigree of family with inherited cryptic translocation of chromosomes 4 and 11. The proband is individual V-11. Solid symbols represent affected subjects, half solid symbols represent carriers.

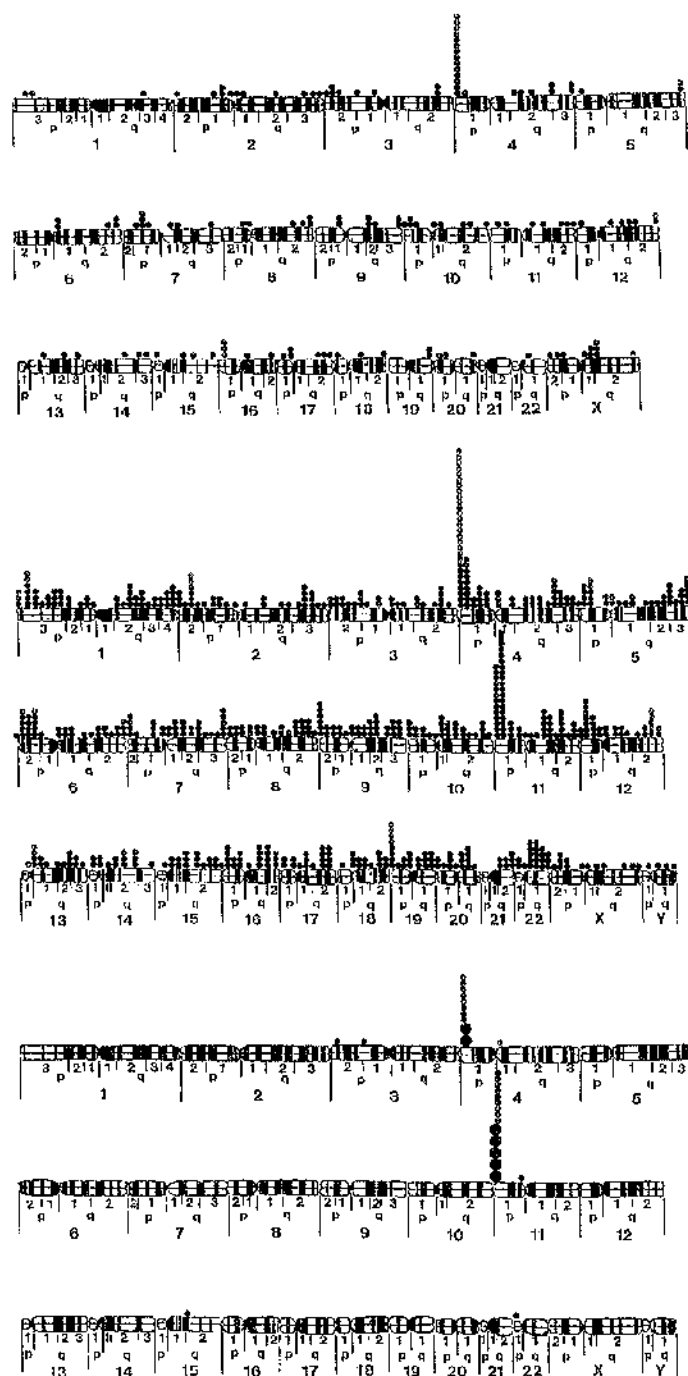


Fig.3-12 Ideograms showing signal distribution following phins311 (11p15.5) or pK082 (4p16.3) ISH to members of a family with inherited cryptic t(4;11).

TOP: 136 signals, with signal peak at 4p16.3, recorded from 27 cells following pK082 hybridisation to IV-12, mother of proband V-11.

MIDDLE: 640 signals, with signal peaks at 4p16.3 and 11p15.5, recorded from 114 cells following pK082 hybridisation to balanced translocation carrier IV-11, father of proband V-11.

BOTTOM: 93 signals, with signal peaks at 4p16.3 and 11p15.5, recorded from 24 cells following phins311 hybridisation to proband V-11. Larger dots represent ten signals. Signal number at distal 4p (28) is approximately half that at 11p (59).

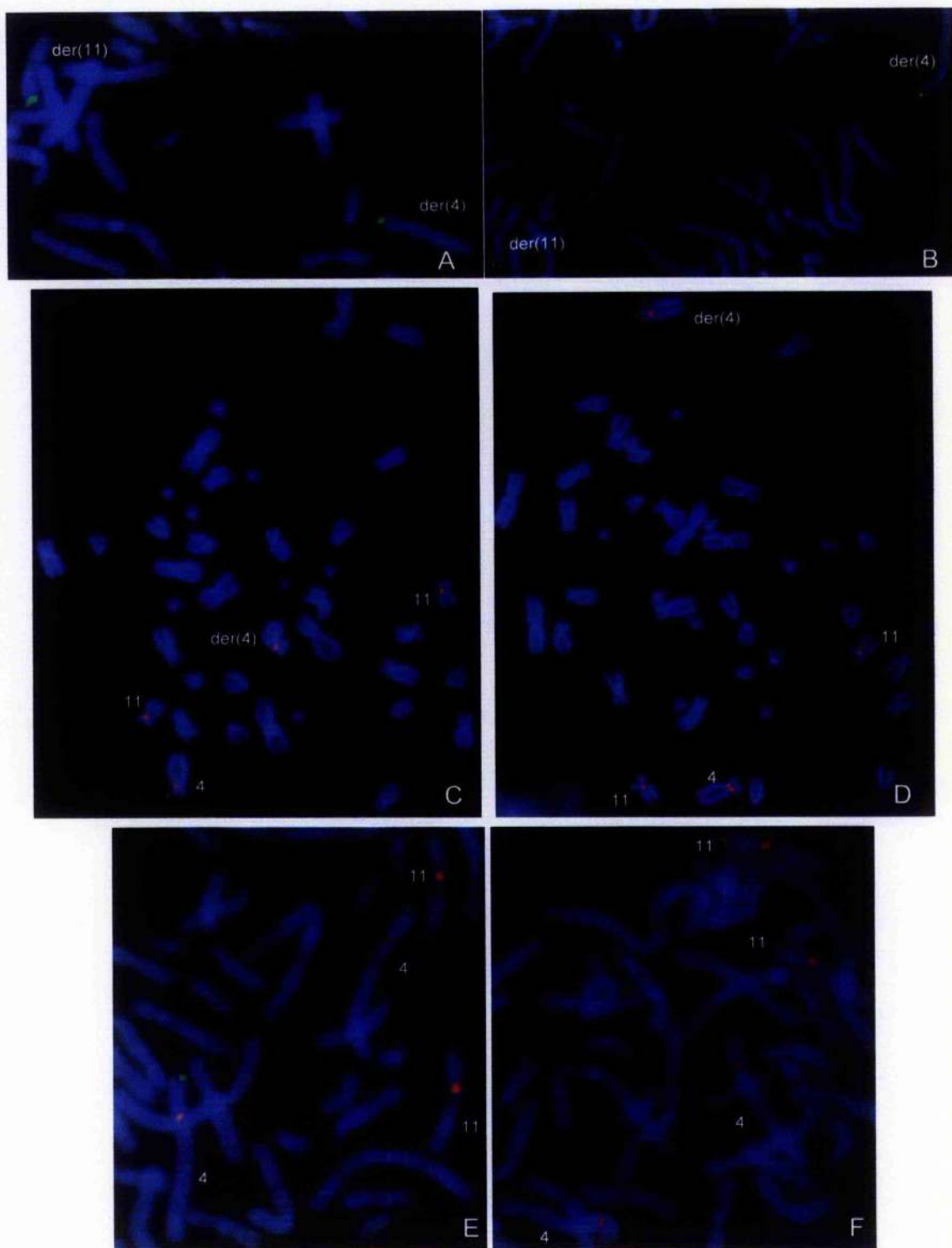


Fig.3-13 Hybridisation of chromosome 4 and 11 probes to a familial t(4;11) translocation. A and B: Hybridisation, of pK082 (4p16.3) and phins311 (11p15.5) respectively, to metaphase spreads from a balanced translocation carrier. Chromosome identification was by reverse DAPI banding (not shown). C and D: Hybridisation of chromosome 4 (C) and 11 (D) telomere probes (Oncor) to CVS metaphases. Chromosome 4 and 11 alpha satellite sequences (Oncor, in red) provide chromosome identification. C shows one copy of 4ptel probe sequence, D shows three copies of 11ptel sequence. E and F: Hybridisation of chromosome 4 (E) and 11 (F) telomere probes (Oncor) to amniotic fluid metaphases. Both chromosomes 4 and 11 show normal signal patterns.

3.2.3 Microdeletion Testing in Rubinstein-Taybi Syndrome

Probe Preparation

A bacterial stab culture containing cosmid RT1 was grown in ampicillin-containing LB broth. The cosmid was then extracted (Circleprep, yield 100ul 0.24ug/ul) and biotinylated by nick translation.

Following a publication reporting microdeletions at the RTS CBP gene locus which would not have been detected with probe RT1 (Petrij et al 2000), DNA from cosmids RT102, RT191, RT203 and RT166, which span the CBP gene, was later obtained and similarly labelled.

Hybridisation Strategy

RT1 Probe: Optimisation experiments were necessary as preliminary ISH with 10 and 20ng/ul probe plus 1ug/ul total human genomic DNA produced excessive background in addition to 16p13.3 signal. These experiments included altering probe and competitor DNA concentration, using Cot1 competitor, and slide pretreatments (ageing at 65°C for 2 hours or incubating in 2xSSC at 37°C for 30 minutes). A probe concentration of 6ng/ul and inclusion of 0.5 or 1ug/ul total human DNA produced acceptable signal strength and background levels without slide pretreatment. Post-ISH washing, at 72°C, in 2xSSC for 5 minutes or 0.4xSSC for 2 minutes was later tested to avoid the use of formamide.

Washing at 0.4xSSC for 2 minutes produced a satisfactory result .

A DIG-labelled chromosome 16 alpha satellite centromeric probe (Oncor) was co-hybridised (0.3ul per 10ul hybridisation mix) to assist chromosome 16 identification, otherwise DAPI bands were enhanced and reversed following digital imaging.

RT102, RT191, RT203, RT166 Probes: Initial ISH with 10ng/ul probe on control metaphases established that the specific signals from these sequences were weak and/or always accompanied by excessive background hybridisation, which rendered optimisation difficult. Repeat labelling and assessment of different probe concentrations (6 to 20ng/ul) and 1ug/ul total human DNA produced preparations of variable quality. Labelling using twice the recommended concentration of DNA did produce some improvement in signal:background ratio. This allowed the probes to be used diagnostically, at 12ng/ul with 1ug/ul total human DNA competitor.

Hybridisation to Patient Samples

Metaphase chromosome preparations from fifteen patients referred with "query RTS" were hybridised to RT1 probe and at least five metaphases with good signal quality

examined in each preparation. All showed signal on both chromosomes 16, indicating absence of deletion at the RT1 locus (Fig.3-10).

It has been possible to test six of the original RTS patient samples with RT102, RT191, RT203 and RT166. No microdeletions have been detected.

3.2.4 Duchenne and Becker Muscular Dystrophy Carrier Studies

Prior to the development of quantitative PCR there was no method in use in this department which could simply and unequivocally diagnose DMD/BMD carrier status. The work described here was performed to instigate such a service. It was then continued to support quantitative PCR testing during the period of its introduction to the diagnostic repertoire of the molecular genetics division.

Isolation of Probes

Twenty nine bacterial cultures carrying different DMD/BMD exon-specific cosmids were first plated out on either ampicillin (the Leiden clones) or kanamycin-containing agar. Three to six colonies were picked from each plate, starter cultures incubated and glycerol stocks prepared from these for storage at -20°C. Initially one or two of these starter cultures or glycerol stocks from each of the twenty nine bacterial cultures were used to inoculate 100ml LB broth for further culture and subsequent cosmid isolation using the Circleprep kit (Bio 101). The DNA yield was typically 0.2-0.5ug/ul (20-50ug). In an attempt rapidly to provide small yields of cosmid isolate for initial ascertainment of probe suitability the Insta-mini-prep kit (5 Prime→3 Prime Inc.) was used with cosmids 7a and e47, but this approach failed to produce signal. It was not possible, with the small amount of DNA produced, to measure the yield therefore quantities for labelling had to be estimated. The use of twice the recommended culture volume in the Insta-prep process and twice as much Insta-prep product in nick translation was also tried without success.

Contamination of Probes

During the course of this study, detection of weak signal on the X chromosome in a low proportion of metaphases from males with confirmed deletion indicated possible contamination of some probes with other exons. Aliquots of probe isolates were therefore sent to the molecular genetics division of this department for testing by multiplex PCR with exon-specific primers (Table 3-17).

Extra colonies were cultured, extracted and hybridised for probes which failed to produce adequate signal or showed contamination with other exons. Although this did result in

contamination-free probe isolate in one case (NRM10/11), the hybridisation efficiency of this probe remained unsatisfactory.

NRM47, NRM51 and NRM52 were among those probes exhibiting contamination. These cosmids were also used in the Wessex Regional Genetics Laboratory, Salisbury, where the presence of contaminating sequences was confirmed. Following selection of subclones of these probes the Wessex Laboratory generously supplied glycerol stock of which e47b, e51b and e52 were 'pure' isolates.

PROBE	CLONE*	CONTAMINATING EXON	EVENTUAL DIAGNOSTIC ** USE
NRM10/11	a	47	no
	b	8	no
	f	none	no
	g	none	no
NRM47	original stock	19, possibly 52	no (on recognition of contamination)
	e47a (S)	45	no
	e47b (S)	none	yes
cMA1G3 (exons 50,51)	a	53	no
	b	53	no
	c	24	no
	d	24	no
NRM51	original stock	19,47,50,52	no (on recognition of contamination)
	e51a (S)	47	no
	e51b (S)	none	yes
NRM52	original stock	19	no (on recognition of contamination)
	e52 (S)	none	yes
cMA1G6 (exon 54)	a	51,53	no
	b	51,53	no

Table 3-17 DMD/BMD cosmid clones with contaminating dystrophin sequences.

* Probes marked (S) were supplied by the Wessex Regional Genetics Laboratory, Salisbury.

** Probes employed diagnostically showed no contamination with other exons and adequate hybridisation efficiency.

Probe Labelling

Isolated DMD/BMD exon-specific probes were labelled with biotin by nick translation and, to assist chromosome identification, co-hybridised with biotinylated chromosome X alpha satellite centromeric probe (DXZ1, Oncor). Addition of 0.8ul of a denatured 1:20 dilution of DXZ1 in hybridisation buffer to 10ul of pre-annealed cosmid probe mix provided adequate centromeric signal.

During these investigations temporary problems with biotin-11-dUTP supply prompted investigation of another labelling approach. The newly-available BioNick kit (Gibco BRL

Life Technologies) was therefore tried with cMA2B3 and e47 probe DNA. This is similar to the standard Gibco nick translation kit but biotin-14-dATP is supplied ready-mixed with the unlabelled nucleotides. Although slight improvement did result from the use of twice the recommended DNA concentration in the BioNick reaction the signal obtained from these cosmids was weak, so the original labelling approach was re-adopted when biotin-11-dUTP supply recommenced.

Optimisation of Probe Hybridisation

Prior to diagnostic use, probes were assessed at a range of concentrations (10-20ng/ul) for hybridisation efficiency and background level. Most of the satisfactory probes, i.e. those which produced signals on both X chromosomes in at least 80% of metaphases, were used at a concentration of 20ng/ul.

Initially an incubation temperature of 42°C was used for hybridisation but this was later reduced to 37°C to improve weaker probe signals.

An extra round of amplification was attempted for several of the weaker probes but, because of increased background, this produced a consistent improvement with only one cosmid, cYD4.66. Extra amplification appeared to be most beneficial if target metaphases were membrane-bound.

Although probes were assessed with both competitor DNAs, those originating from Leiden were found to work better or as well with Cot1 DNA and those supplied by Manchester worked better or as well with total human genomic DNA. Either Cot1 DNA at 0.2ug/ul or total human genomic DNA at 1ug/ul was used for all probes except NRM17 (0.25ug/ul Cot1), NRM19 (0.23ug/ul Cot1), cYD4117 (0.4ug/ul total human genomic DNA) and cMA2F5 (1.25ug/ul total human genomic DNA).

Post-hybridisation stringency washing was initially two 5 minute treatments in 50% formamide at 42°C, with a shorter wash attempted for weaker probes. Later in the study all probes were washed for 2 minutes in 0.4xSSC at 72°C.

Following ISH efficiency and contamination testing, twenty probes proved to be of acceptable quality for diagnostic use (Table 3-18). Adequate hybridisation was however only achieved with cosmid cYD4117 by using twice the normal concentration of DNA for labelling, 400ng probe and just 0.4ul total human DNA per area, and reducing wash stringency. This probe was only used on one patient who had a male relative with an exon 44 deletion.

PROBE	LOCUS	% OF ALL EXON DELETIONS DETECTABLE ¹	ORIGIN ²	COMPETITOR DNA	COMPETITOR CONCENTRATION
NRM1 <i>c104C038</i>	exon1	1	M	Cot1	0.2ug/ul
NRM3 <i>c104A1047</i>	exon3	8	M	Cot1	0.2ug/ul
cLA1A5	exon3/ exon4	8 9	L	total genomic	1ug/ul
NRM7 <i>c104E0790</i>	exon7	10	M	Cot1	0.2ug/ul
NRM8 <i>c104F02123</i>	exon8	10	M	Cot1	0.2ug/ul
cLA1F2	exon8/ exon9	10 10	M	total genomic	1ug/ul
NRM13 <i>c104F0393</i>	exon13	11	M	Cot1	0.2ug/ul
NRM17 <i>c104D02129</i>	exon17	9	M	Cot1	0.25ug/ul
NRM19 <i>c104B0281</i>	exon19	9	M	Cot1	0.23ug/ul
cYD4117	exon44	7	L	total genomic	0.4ug/ul
cPT4	intron44	N/A	L	total genomic	1ug/ul
cAL24	intron44	N/A	L	total genomic	1ug/ul
cPT1	intron44/ exon45	N/A 37	L	total genomic	1ug/ul
cYD4.66	intron44 (3'P20)/ exon45	N/A 37	L	total genomic	1ug/ul
NRM47/e47 <i>c104E0143</i>	exon46/ exon47	41 41	M/S	Cot1	0.2ug/ul
cMA2B3	exon48/ exon49	37 34	L	total genomic	1ug/ul
cMA2F5	exon49/ exon50	34 33	L	total genomic	1.25ug/ul
NRM51/e51 <i>c104C0461</i>	exon51	26	M/S	Cot1	0.2ug/ul
NRM52/e52 <i>c104A1287</i>	exon52	23	M/S	Cot1	0.2ug/ul
cMA1C4	exon55	7	L	total genomic	1ug/ul

Table 3-18 DMD/BMD cosmids used diagnostically.

N/A: Not applicable - the probe is an intronic sequence. Intron 44 is the intron with the most deletion breakpoints.

¹The percentage of all DMD/BMD exon deletions which involve the exon sequence in this probe. Information obtained from http://www.dmd.nl/DMD_deldup_Leiden.html

²M=Manchester, S=Salisbury, L=Leiden.

The ICRF numbers of the cosmids provided by the Manchester group are written in italic beneath the probe names.

Strategy for Diagnostic Testing

Prior to diagnostic use, probes were preferably assessed on affected or known carrier relatives. If the probe failed to produce signal on the deleted chromosome X it was considered informative and suitable for carrier determination in that family. Carriers showed this probe signal at Xp21 on only one of their X chromosomes, whereas both X chromosomes showed signal in non-carriers (Fig.3-14).

Ten metaphases were analysed for each test with a normal result, twenty to twenty-five if abnormal. ISH was repeated, with normal controls, for all apparently deleted or carrier cases.

Diagnostic Results

One hundred and twenty one females were tested, using appropriate cosmids from the optimised panel of twenty probes, to determine DMD/BMD carrier status (Table 3-19). In the majority (eighty five) of these investigations, the results of previous DNA studies indicated the appropriate exons for FISH testing and the informativeness of a FISH probe could be confirmed by hybridisation to a known carrier or affected male relative. One individual (SC), in whom carrier status was excluded, had previously been classified as a carrier by CK levels.

Another FISH result might have indicated possible gonadal mosaicism - an allegedly obligate carrier (MW) was not deleted at exon 7, unlike her son. Unfortunately the other reputedly affected male relative was not available for testing. FISH also identified a deletion at exon 52, in carrier SE, which was missed by quantitative PCR. The FISH result was confirmed by further molecular studies at another centre.

Seven females were referred for testing but FISH studies on their male relatives, who had exon deletions identified by molecular means, showed the available FISH probes to be uninformative.

In nineteen cases, no relative was available for FISH confirmation and the family deletion was recognised by DNA studies alone. In these cases, failure to detect hemizygosity in the test female by FISH was not considered to exclude carrier status unless the deletion defined by molecular means was at least one exon longer at each side than the FISH probe sequence used (e.g. the deletion included exons 48-52 and the FISH probe used was specific for exons 49/50). Carrier status could then be provisionally excluded.

Seventeen of the females tested had no available affected or carrier relative to allow identification of deletion (or confirmation that dystrophin deletion was the cause of the muscular dystrophy in their family). Nineteen of the twenty-probe series (excluding

cYD411.7) had to be used in the FISH investigations on this group. This did achieve detection of deletions in three of these individuals:

- JG (cPT1, cAL24, cPT4 deletion), who later had PND by FISH on cultured amniocytes. The female fetus was also a carrier.
- MB (e52 deletion). Determination of this deletion also allowed exclusion of carrier status in her daughter and another female relative.
- EB (NRM51 deletion), although signal was present on both chromosomes X in 1/20 metaphases. This could have been due to background, the contamination of the probe with exon 52 DNA (and no deletion at exon 52) or to only partial deletion of NRM51 sequence. No material remained for subsequent testing with 'pure' exon 51 probe.

In addition to the fifty two male patients tested to assess the suitability of particular FISH probes for carrier detection, two were tested for inheritance of a familial BMD deletion (Table 3-20). FISH confirmed deletion in one of these individuals.

In total eighty three families were investigated with DMD/BMD cosmids.

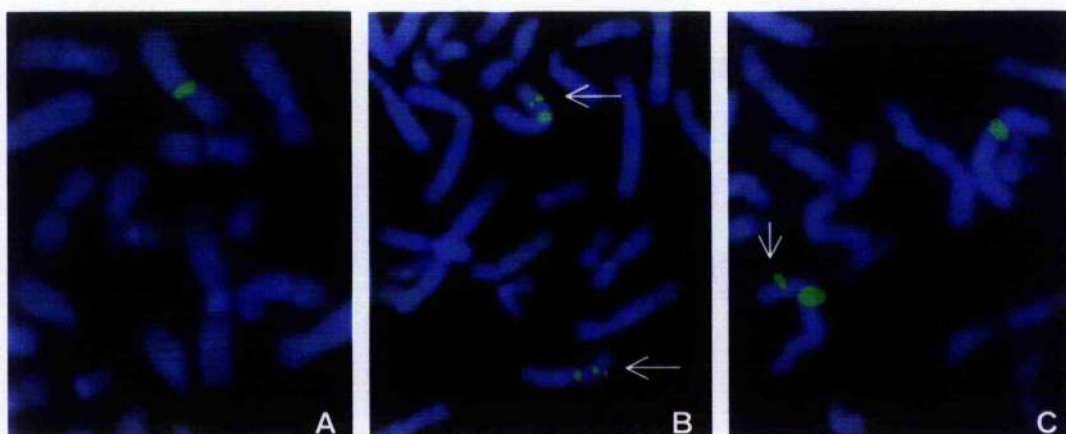


Fig.3-14 DMD/BMD carrier determination by FISH with dystrophin exon-specific probes. A: Metaphase from a DMD patient following hybridisation to a dystrophin exon probe which detects the deletion in this individual. No signal is present at Xp21. The X centromere is highlighted with an X alpha satellite probe to aid chromosome identification. B: Metaphase from a female relative of this DMD patient following hybridisation to the same dystrophin exon-specific probe. Signal at Xp21 (arrowed) is present on both chromosomes X, indicating that this individual is not a carrier of the familial deletion involving this sequence. C: Metaphase from another female relative of this DMD patient following hybridisation to the same dystrophin exon-specific probe. Signal at Xp21 (arrowed) is present on only one chromosome X, indicating that this individual is a carrier of the familial dystrophin deletion involving this sequence.

METHOD OF IDENTIFICATION/CONFIRMATION OF FAMILIAL DELETION	NUMBER REFERRED	DELETION/CARRIER STATUS		PROBES UNINFORMATIVE
		CONFIRMED	EXCLUDED	
FISH on a male relative	77	21	52 (one a possible GM)	4
FISH on a female carrier	8 (one PND)	2	6	0
DNA studies then FISH on male relative, but FISH uninformative	7	0	0	7
DNA studies only	19	6	7 (provisional)	6
DMD/BMD unconfirmed/ deletion uncharacterised (affected male unavailable for testing)	17	3	0	14
Total number of potential female carriers referred	128	32	65	31

Table 3-19 Confirmation or exclusion of carrier status achieved by FISH on female relatives of affected males. GM:gonadal mosaic

REASON FOR FISH TESTING	NUMBER TESTED	DELETION DETECTED	
		YES	NO
To assess suitability of FISH probe for carrier testing	52	43	9
To confirm affected with BMD (FISH probes informative)	2	1	1
Total number of males tested	54	44	10

Table 3-20 Numbers of male patients tested using FISH with DMD/BMD probes and reasons for testing.

3.2.5 Screening for Cryptic Subtelomeric Rearrangements

As a consequence of the report that around 6% of patients with idiopathic MR might have cryptic subtelomeric rearrangement (Flint et al 1995), contact was made with this group which was then attempting to isolate efficient, specific FISH probes for all subtelomeric regions. A series of cosmids was received for assessment as part of this effort.

The probes which the group isolated (Ning et al 1996) were then used in the development of the commercial Multiprobe-T device (Cytocell), designed to allow diagnostic screening for subtelomeric rearrangements. The first part of the study described here concerns a contribution to these developments.

The subsequent introduction of the Multiprobe-T device allowed diagnostic screening of individuals with idiopathic MR to commence. Its use, also reported in this study, laid the foundations of a diagnostic service and afforded an initial assessment of the incidence of subtelomeric rearrangements in Scottish patients with idiopathic mental retardation.

Assessment of Subtelomeric Cosmids for Specificity and Hybridisation Efficiency

Eight bacterial culture plates were received from the Institute of Molecular Medicine, John Radcliffe Hospital, Oxford. Two contained scos cosmids subcloned from unidentified telomeric YACs (2067 and D3) and six had subclones from chromosome 1q, 2q, 3q, 8q, 9q, and 18q telomeric YACs. For each plate a gridded 'plan' had been provided on which was noted the positions of those colonies which had hybridised to Cot1 DNA and therefore contained human rather than purely YAC DNA.

In total, 140 colonies carrying cosmids with human DNA inserts were picked and incubated as 5ml starter cultures in ampicillin-containing broth. Cultures which failed to grow were re-picked. Glycerol stocks were prepared from the 69 cultures which produced growth at the first or second attempt. By the time a set of subtelomeric probes had been completed by the Oxford group, cosmid DNA had been extracted from 18 of these 69 glycerol stocks (using a Qiagen kit) and 16 of these cosmid extracts with strong bands on electrophoresis had been DIG-labelled and hybridised to normal metaphase preparations at 10-15ng/ul with 1ug/ul total human DNA competitor. DIG, rather than biotin, label was chosen because of the shorter detection protocol. Extractions were repeated for some cosmids with low yield and no signal on FISH (e.g. 2112.2b2), in one case using a colony from a duplicate plate (2053.2d4). If only weak FISH signals were present with any cosmid (e.g. 2123.2d1, 2050.2d2) further trial hybridisations were performed. These involved changing, separately, the cosmid concentration (to 20ng/ul), the DNA competitor (to Cot1) or the total human competitor DNA concentration (to 0.5ug/ul). It was noted that 200ng/ul Cot1 was a less effective competitor than 1ug/ul total human genomic DNA.

Where excess signals were present (e.g. D3g5), the cosmid concentration used was reduced to 5ng/ul. None of the hybridised cosmids produced signal of adequate strength or specificity (Table 3-21) and only two (2112.2b2 and 2123.2d1) produced clear signal on the chromosome telomere to which they were expected to hybridise.

Lack of success with other cosmid clones occurred at various stages in the cosmid extraction and ISH process. Failure of some colonies to grow in broth might have occurred because they had died in transit, or because they were picked from plates which were not freshly streaked, which can lead to loss or mutation of construct. Also, it was recognised in advance that not all colonies would grow well, and that this depended on the cosmid insert (Dr Flint, personal communication).

Cosmid inserts which produced no, or only very weak, FISH signal may have had a high proportion of repetitive sequences, and these were 'competed out' during CISS ISH. Cross hybridisation to many chromosomes (as seen with 2123.2c5 and 2053.2b5) is likely to have arisen because of the inclusion, in the cosmid insert, of distal subtelomeric sequences which are common to many chromosomes (as explained in Knight and Flint 2000). The presence of more proximally located non-unique subtelomeric sequences is likely to have been the cause of some cosmids (such as 2112.2b2 and 2050.2d2) cross hybridising to a few chromosomes (Knight and Flint 2000). Homology of 2q and 8p telomeric sequences had been recognised previously (Dr Flint, personal communication), which explains the cross hybridisation (to 8p) of 2qtel sequences in cosmid 2112.2b2. This work excluded the studied cosmid sequences as suitable probes.

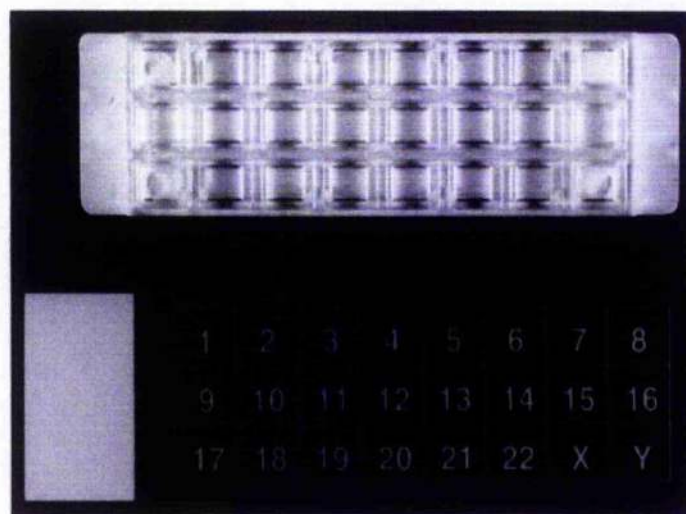


Fig.3-15 The Multiprobe-T device.

YAC	TEL	STARTER CULTURES	GLYCEROL STOCKS	COSMIDS PREPARED	[yield]	FISH RESULTS
2112.2	2q	3	1	b2 repeated	[22ug]	2q and 8p
2123.2	1q	20	14	c5 repeated	[27ug]	nonspecific
				d1	[13ug]	1q, 1p, few other telomeres
				e1	[1.1ug]	no signal
				g3	[1.3ug]	no signal
2050.2	18q	21	7	e6	[8ug]	weak, nonspecific
				d2 repeated	[25ug]	Bgrp, 10q, 1cen, acro p arms
				c4	[35ug]	Bgrp, 10q, 1cen, acro p arms
				a5	[17ug]	no signal
2053.2	8q	12	1	b5	[7ug]	nonspecific
				d4 repeated	[2ug]	no signal from either original or duplicate plate
2175	3q	25	21	none		FISH not performed
2048	9q	16	8	a3	[1.3ug]	no signal
				b6	[1.9ug]	no signal
				c4	[6ug]	not hybridised-weak gel bands
				f1	[6ug]	not hybridised-weak gel bands
2067	?	3	3	a1	[4ug]	no signal
D3	?	39	8	g5	[15ug]	nonspecific
				h3	[35ug]	nonspecific

Table 3-21 Hybridisation results with cosmid subclones of telomeric YACs.

Bgrp: B group chromosome; acro: acrocentric chromosome; cen: centromere

The Cytocell Multiprobe-T Device

The Multiprobe-T is a disposable device which allows the independent hybridisation of each of a 41 telomere probe set to a fixed metaphase preparation spotted on a single template microscope slide (Fig.3-15). There are no probes for the short arms of acrocentric chromosomes. The device consists of a 2.5 x 6.5cm array of raised square panels each of which carries one (in the case of the acrocentric probe panels) or a pair of telomeric probes. Hybridisation solution is placed on each of the panels then both probe and target DNA on the template slide are denatured simultaneously under the device when the slide is heated to the denaturing temperature. This technology had previously been applied to sets of whole chromosome paints (the Multiprobe-M) and centromeric probes (the Multiprobe-I). Involvement, in 1995, with evaluation of the original plastic Cytocell Multiprobe-M, and subsequently of a glass device with directly labelled paints, led the company to request assistance with the assessment of a prototype Multiprobe-T in October 1997. Generally poor results were obtained with this plastic device and twelve of the probes failed to produce signal on one sample. It was decided, from the results of the Multiprobe-M assessments, that the use of glass rather than plastic allowed more even

contact of device and template slide and prevented 'drying out', so a glass version of the Multiprobe-T was commercially launched in early 1998.

During the course of this investigation the device specification altered. It initially carried indirectly labelled probes requiring detection with fluorescent antibodies (this version was used on 59 cases in this study). In 2000 this was superseded by a device with directly labelled fluorescent sequences (used on 41 cases). This change to direct labelling was made feasible by the replacement of most of the original cosmid probes with more robust PAC, P1 or BAC clones (Knight et al 2000).

Multiprobe-T Protocol Modifications

The Multiprobe-T device was used broadly according to the manufacturer's instructions and as described by Knight et al (1997), however the recommended protocol had to be slightly modified for use on the cytogenetic preparations produced in this department. Alterations included prior denaturation of the test slide in 70% formamide, prolonged pre-warming (for over twenty minutes) of device plus slide combination prior to co-denaturation, and altered co-denaturation time. Version 1 of the manufacturer's method stipulated five minutes denaturation, but this was reduced to two to three minutes for this study to avoid damage to chromosome preparations. This was communicated to the manufacturer, and later versions of the recommended protocol advised denaturing for two minutes.

It was found that, when dropping fixed samples on to the template slide, pre-warming of the slide in a humid atmosphere over a water bath at 65°C produced an improvement in the chromosome spreading obtained from poorer quality samples.

Testing with Cytocell Multiprobe-T for the Detection of Cryptic Subtelomeric Rearrangements in Idiopathic MR

Employing the modifications described above, the device was used to study a series of 100 patients to assess the incidence of subtelomeric rearrangement.

Patients

Eighty eight unrelated individuals with unexplained moderate or severe MR, eight with mild or mild to moderate MR, and four with mild to moderate MR plus behavioural difficulties (a total of 55 males and 45 females) were selected for subtelomere screening based on clinical examination and family history by consultant clinical geneticists in Glasgow, Aberdeen, and Newfoundland, Canada.

Retrospective examination of genetic records revealed that thirty four patients presenting with moderate to severe MR and three patients with mild to moderate retardation had a family history of MR. All patients with moderate to severe MR and eleven of the twelve individuals with mild MR had been assessed as having at least one physical abnormality, as summarised in Appendix II.

Fixed chromosome suspensions were prepared by standard cytogenetic methods from lymphocyte or fibroblast cultures. All patients had initially undergone other cytogenetic investigations with normal results. These included karyotyping at 550 band resolution to exclude overt chromosome abnormalities and FISH testing for microdeletion at the DG/VCFS (5 patients), Williams syndrome (3 patients), Smith-Magenis syndrome (2 patients), RTS (3 patients) and retinoblastoma (1 patient) loci.

Analysis

To verify the reliability of the Multiprobe-T device and its analysis, three samples from cases of recognised cryptic abnormality were anonymised and tested with the first 46 patients to be screened. Two of these three samples were from individuals with cryptic $t(2;17)$, the third had cryptic $t(4;20)$. These familial rearrangements had previously been identified by CGH (Ghaffari et al 1998).

Analysis of the 100 patients in the test series involved examination of at least three metaphases in each probe area. Images of two of these metaphases were stored and independently checked for result confirmation.

In seventy nine cases screened further analyses had to be performed using individual chromosome subtelomere probe sets (Chromoprobe-T, Cytocell) because of inadequate hybridisation quality in some of the Multiprobe-T device areas. An average of 3.8 probes had to be repeated for 59 indirectly-labelled devices, ranging from 0 probes (in six cases) to 19 (in one case). In three of the four cases with ten or more failed probes a second device was used for repeat testing. With directly-labelled devices, an average of 2.5 probes per device were repeated, range 0 (in fifteen cases) to 16 (in one case with a very poor quality metaphase preparation).

As the manufacturer advised, and as reported (Knight and Flint 2000), cross hybridisation involving several of the subtelomeric regions was occasionally observed. Probe selection had necessarily involved a compromise between sensitivity and specificity - more distal sequences are most sensitive to small rearrangements but are more likely to include nonspecific elements. In particular, cross hybridisation of 8p sequence to 1p, not recognised by the device manufacturer until the third version of the direct label protocol

was released, was detected in 10 cases tested with the direct device. No polymorphic deletions of 2q sequence were observed in this series.

Three rearrangements, detailed below and in Table 3-22, were detected in the test series of 100 affected individuals and the cryptic rearrangements included as positive controls were correctly identified.

Subtelomeric Rearrangement in Patient DR

This boy with severe MR, oculomotor apraxia and mild facial dysmorphism was shown to have an unbalanced subtelomeric rearrangement involving a derivative chromosome 9 with deletion of the subtelomeric region of the short arm including probe sequence 43N6, and additional material from the subtelomeric region of the long arm of chromosome 3 including sequence D3S1272, karyotype:

46,XY.ish der(9)t(3;9)(q29;p24)(wcp3+,D3S1272+,43N6-,D9S2168+). This result (Fig.3-16) was confirmed with another subtelomeric probe for chromosome 3 long arm (D3S1445/D3S1446, believed by the manufacturer, Oncor, to lie proximal to the Cytocell 3q probe but less than 450kb from telomere). Whole chromosome 3 paint (Cambio) showed chromosome 3 material on the derivative chromosome 9 in all 10 cells examined, including one metaphase with very short chromosomes of approximately 300 bands, and chromosome 9 paint showed an unpainted region on the derivative 9 in all of 15 metaphases. Paint endpoints on reverse DAPI banded images allowed the estimation of translocation breakpoints as 9p22.3~p23 and 3q27.3~q28. ISH of chromosome 3 and 9 subtelomere probe sets to chromosome preparations from the child's parents showed that the rearrangement was *de novo*.

Subtelomeric Rearrangement in Patient AS

A male baby with TOF and developmental delay, and subsequently his affected uncle (DS), were shown to have partial monosomy of chromosome 9q (involving probe sequence D9S2168) and partial trisomy 16q (involving sequence D3b1) (Fig.3-17).

Whole chromosome paint could demonstrate a trace of chromosome 16 material on the derivative chromosome 9 in seven of ten metaphases examined. ABL probe sequence (9q34, Vysis) was present on both chromosomes 9. The karyotype was recorded as: 46,XY.ish der(9)t(9;16)(q34;q24)(43N6+,wcp9+,D9Z1+,ABL+,D9S2168-,wcp16+,D3b1+).

Hybridisation of chromosome 9 and 16 subtelomere probe sets to chromosomes from the father of AS showed that he carried the balanced reciprocal translocation involving chromosomes 9 and 16 (Fig.3-17).

Despite careful and focussed scrutiny of the chromosomes 16 in this carrier, whole chromosome 9 paint revealed chromosome 9 material on the derivative 16 (representing the monosomic region in AS) in only one out of ten metaphases.

When the mother of AS became pregnant the prior identification of this familial chromosome rearrangement allowed PND. Individual chromosome 9 and 16 telomere probes hybridised to a CVS preparation showed no rearrangement of these sequences in the foetus (Fig.3-17).

Subtelomeric Rearrangement in Patient MC

This male patient, aged 18 years, presented with severe MR and multiple congenital abnormalities (detailed in Table 3-22). His brother (SC, 21 years) was similarly affected but with VSD, no pulmonary stenosis, scoliosis or hypospadias. Another, deceased, brother had similar features. Both live sibs were shown to have partial monosomy of the long arm of chromosome 18 (involving sequence D18S1390) and partial trisomy of the short arm of chromosome 10 (involving sequence D10S2488). Chromosome 10 material on the derivative chromosome 18 in patient MC was clearly demonstrable with chromosome 10 paint in all cells (Fig.3-18). The karyotype was recorded as: 46,XY,ish der(18)t(10;18)(p15;q23)(52M11+,D18S1390-,D10S2488+,wcp10+).

Relatives subsequently tested included the similarly affected nephew (DD, Appendix II) and the boys' father (DM) and brother (DE) who both carried the balanced form of this reciprocal translocation. Chromosome 18 paint showed translocated material on one chromosome 10 in all cells of carrier DE (Fig.3-18).

In summary, the three different unbalanced subtelomeric chromosome rearrangements detected were all found in the group of patients with severe to moderate learning difficulty or developmental delay, making the prevalence of subtelomeric rearrangements in this cohort of eighty eight unrelated individuals 3.4% (95% CI 0.7%-9.6%).

All three probands had dysmorphic features. Two had a family history of learning difficulty, inheriting an unbalanced form of a parental balanced translocation. A *de novo* rearrangement was found in the third patient, who had an eye movement disorder and premature puberty. Re-examination of 550-band chromosomes was possible for patients DR and DS, the affected uncle of AS, and revealed no apparent karyotype abnormalities. None of the 12 individuals with mild MR exhibited subtelomeric rearrangement.

Update of Subtelomeric Screening Results

Subtelomeric screening has continued as part of the diagnostic service at the Duncan Guthrie Institute. The Totelvysion kit (Vysis), which allows larger hybridisation areas, has

been used instead of the Multiprobe-T device for cases with very low mitotic index. By the end of 2003 a further 107 cases had been tested (of which two were noted to have only mild MR) and 12 produced abnormal results.

Six of these abnormalities were considered unlikely to be clinically significant, being cases of 2q subtelomeric sequence D2S2986 deletion with no deletion of 2q subtelomeric sequence D2S447, suggesting 2qtel polymorphism. In one case the polymorphism could also be demonstrated in the patient's father, in another it was present in an affected twin. The four cases believed to be clinically significant comprised two with syndrome-associated abnormalities (del(1)(p36.3), del(22)(q22.3)), one with a der(18) representing the unbalanced product of a translocation between chromosomes 18 and 20 (der(18)t(18;20)(q23;q13.3)), and one *de novo* del(6)(q27) which includes the TATA box-binding protein (TBP) gene (as determined by FISH with a TBP DNA probe (Oncor)). The clinical significance of the remaining abnormal subtelomere screens could not be conclusively determined. A 7p deletion detected was also present in the patient's mother who had a much milder phenotype. This could be a pathogenic abnormality but with variable expression, or the mother's mildly abnormal phenotypic features could be coincidental. The other abnormality in this category included a case of 8p deletion which was also present in the patient's unaffected father.

Two suspected carriers of subtelomeric deletion were also tested in the absence of material from affected relatives. No abnormalities were found.

Combining these results with those from the initial cohort of 100 patients suggests that the detection rate of clinically significant subtelomeric rearrangement in patients clinically determined to be appropriate candidates for subtelomeric screening lies between 3.4 and, when the 7p and 8p deletions are included, 4.3%. If the patients known to have only mild MR are excluded, giving a population of 193, then the detection rate is 3.6% (95% CI 1.47-7.3) to 4.7% (CI 2.15-8.67%).

KARYOTYPE	DEGREE OF MR	PHENOTYPE
46,XY,ish der(9)t(3;9)(q28;p23) (wcp3+,D3S1272+,43N6-,D9S2168+) <i>de novo</i>	moderate/severe	Cogan's oculomotor apraxia, Duane's ocular retraction, premature puberty
46,XY,ish der(9)t(9;16)(q34;q24) (43N6+,wcp9+,D9Z1+,ABL+,D9S2168-, D3b1+,wcp16+)pat	moderate/severe	tetralogy of Fallot developmental delay
46,XY,ish der(18)t(10;18)(p15;q23) (52M11+,D18S1390-, D10S2488+,wcp10+)pat	severe	pulmonary stenosis, scoliosis, hypospadias, microcephaly, hypoplastic midface, short stature, gastro-oesophageal reflux, asthma, cholelithiasis, joint laxity, hypotonia, reduced pigment

Table 3-22 Subtelomeric rearrangements detected using FISH.

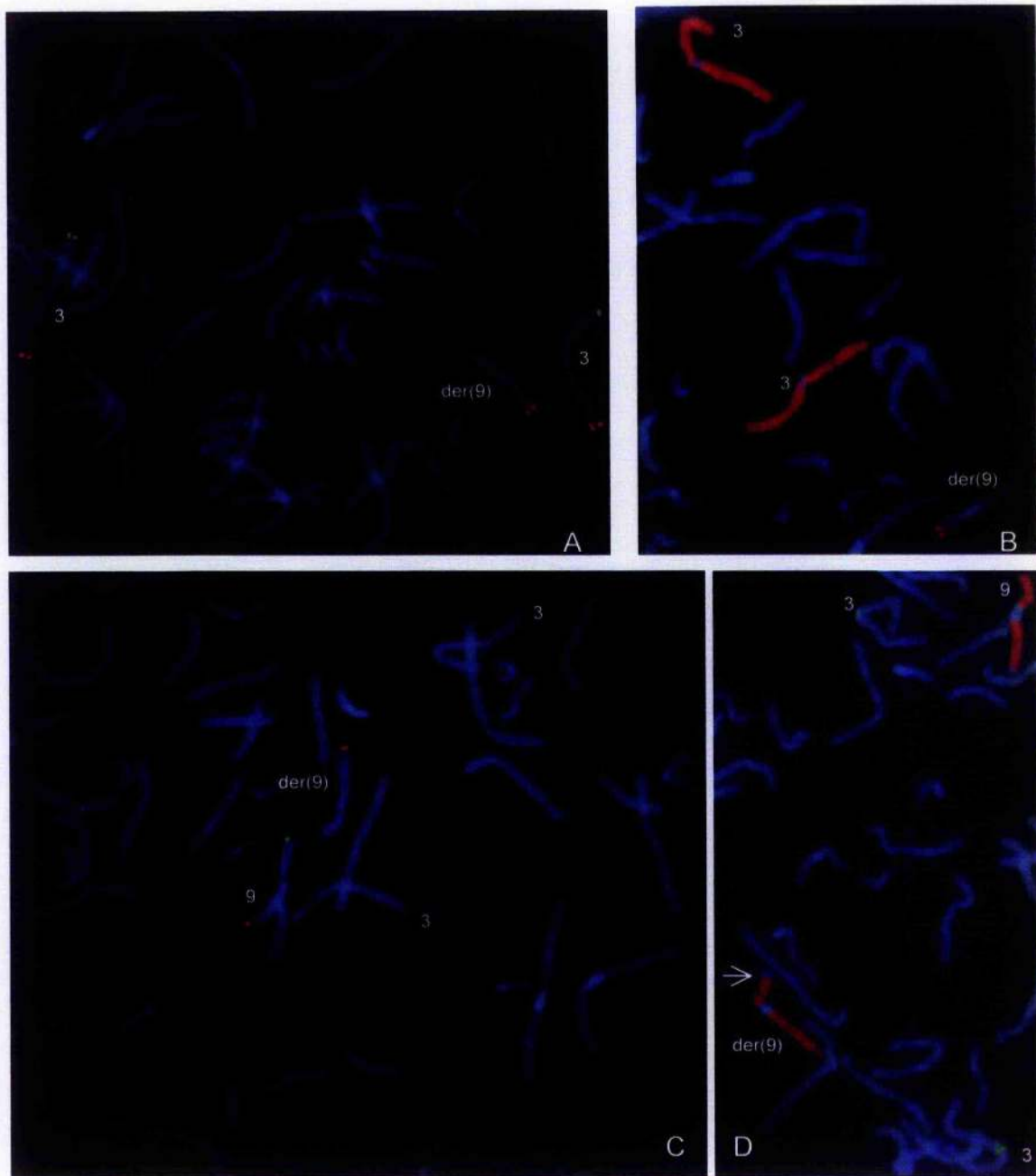


Fig.3-16 Identification of der(9)t(3;9) in individual DR using FISH.

A: Hybridisation to 3p (green) and 3q (red) telomere probes (Cytocell Multiprobe-T) showing an extra 3qtel signal on a derivative chromosome 9.

B: Hybridisation to chromosome 3 paint showing complete paint coverage of both chromosomes 3 and signal on the distal short arm of the derivative chromosome 9.

C: Hybridisation to 9p (green) and 9q (red) telomere probes (Cytocell Multiprobe-T) showing deletion of one 9ptel sequence.

D: Hybridisation to chromosome 9 paint showing an unpainted region due to the translocated chromosome 3 material (arrowed) on the derivative chromosome 9.

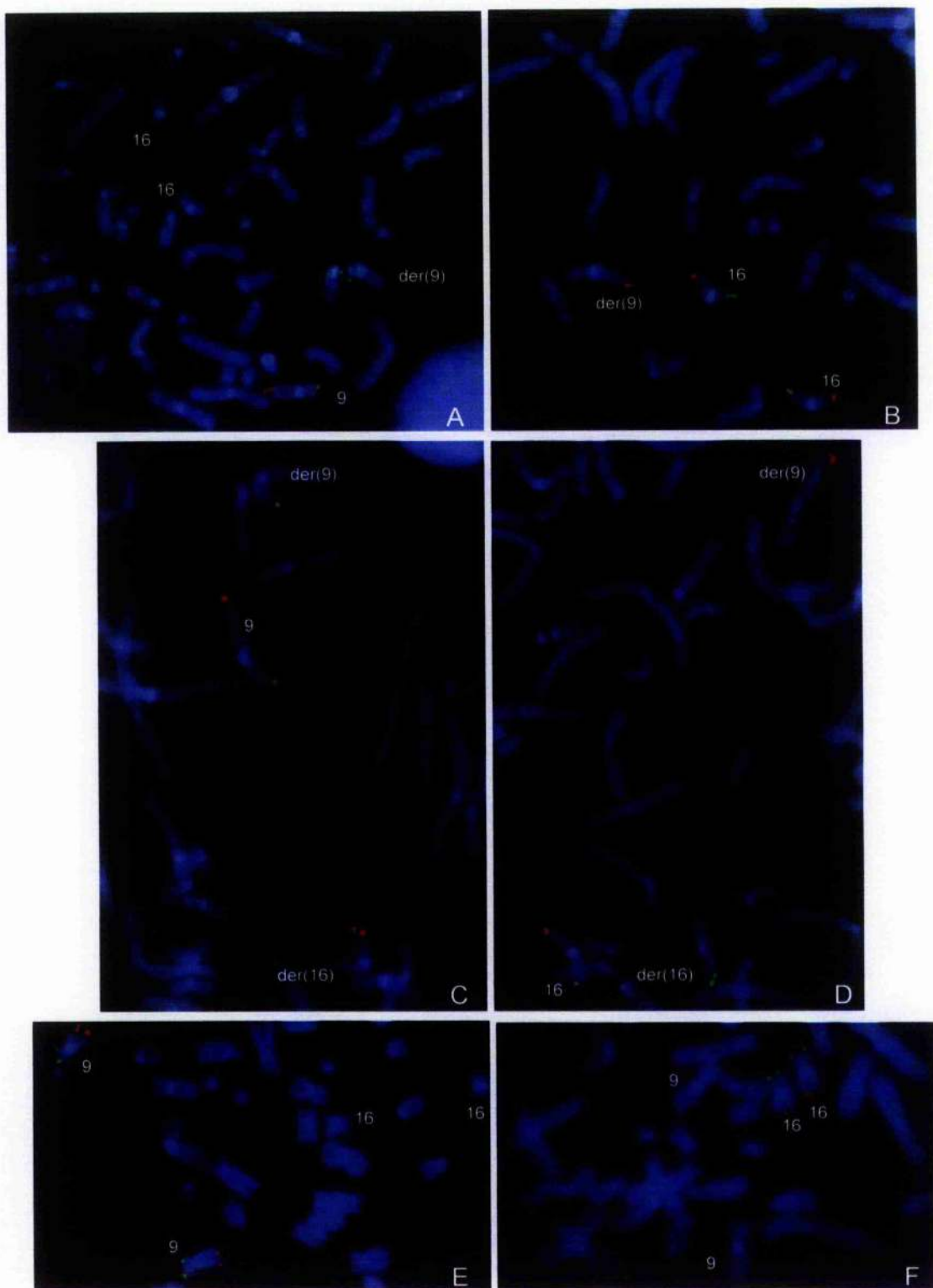


Fig.3-17 Identification of a familial t(9;16) using FISH.

A and B: Hybridisation to 9p and q (A) and 16p and q (B) telomere probes identifies, in affected individual AS, a derivative chromosome 9 with deletion of 9qtel sequence and an extra copy of 16qtel sequence.

C and D: Hybridisation to 9p and q (C) and 16p and q (D) telomere probes identifies derivative chromosomes 9 and 16 in carrier KS, the father of AS.

E and F: Hybridisation to 9p and q (E) and 16p and q (F) telomere probes shows chromosomes 9 and 16 with normal signal patterns in a CVS from the wife of KS.

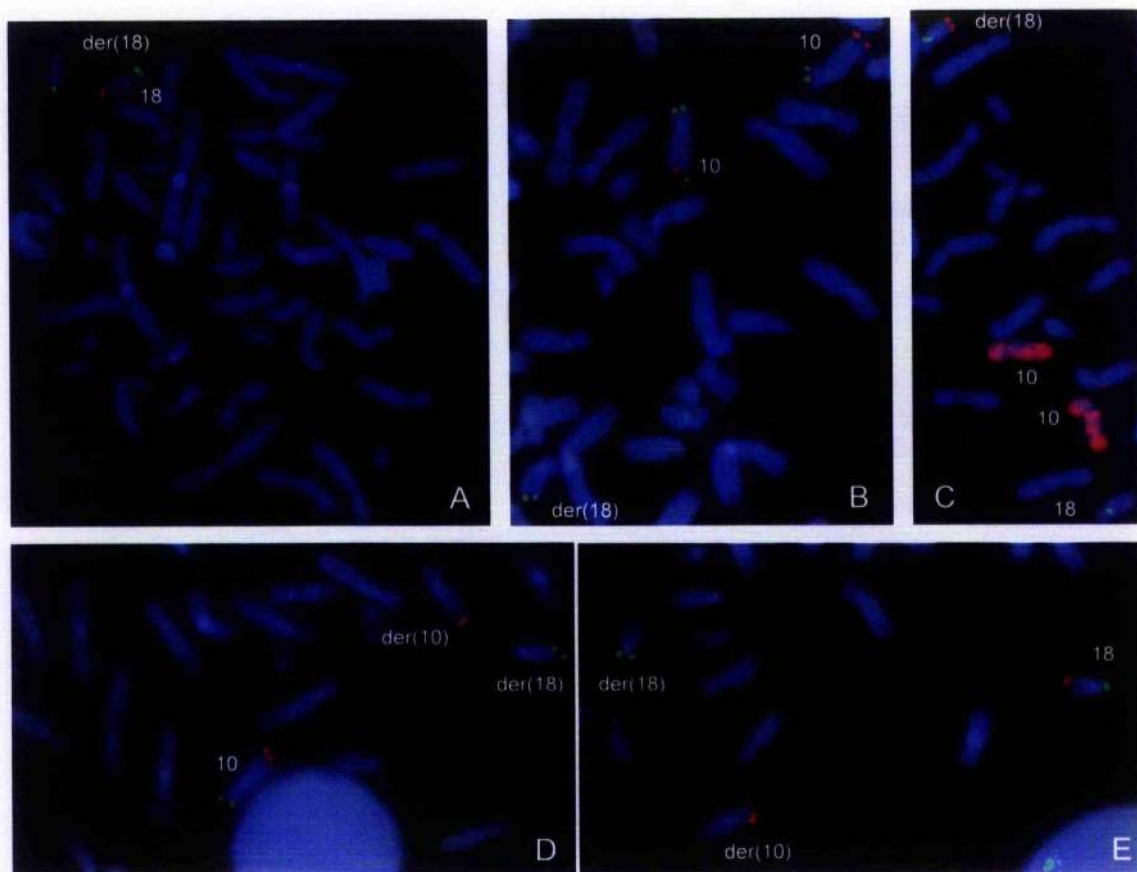


Fig.3-18 Identification of a familial t(10;18) using FISH.

A: Hybridisation to chromosome 18 p and q telomere probes detects deletion of the 18qtel sequence from the derivative chromosome 18 in affected individual SC.

B: Hybridisation to chromosome 10 p and q telomere probes detects an extra copy of 10ptel sequence on the derivative chromosome 18 in affected individual SC.

C: Hybridisation to chromosome 10 paint (red) and 18 alpha satellite probe shows chromosome 10 material on the der(18) indicating, as it is detectable using paint, that this translocated region is likely to be >5Mb in size.

D and E: Hybridisation to p and q telomere probes for chromosomes 10 (D) and 18 (E) identifies derivative chromosomes 10 and 18, indicating that this brother of SC carries the rearrangement in the balanced form.

3.3 Comparative Genomic Hybridisation

CGH provides a global approach for the determination of quantitative genomic change in cases of apparent but unresolvable chromosomal abnormalities. Its sensitivity, approximately that of chromosome painting, also enables it to disclose some subtle or cryptic abnormalities hidden by conventional banding. The work described here involves the development of this technique for diagnostic use and its application to such cases.

3.3.1 Preliminary CGH Experiments Using Indirectly Labelled DNA

The first CGH attempted in this study, performed primarily to gain experience of the procedure, utilised DNA indirectly labelled with biotin and DIG and detected with FITC and Texas Red conjugates. This approach was used for hybridisations of 46,XY and 45,X0 versus 46,XX reference DNA. These experiments established a working DNA concentration for CGH with indirect labelling of 120-300ng per area (published values ranged through 60ng (Kallioniemi et al 1992b), 120ng (Kallioniemi et al 1994), to 1ug (du Manoir et al 1993)). They also showed that pretreatment with 0.2ug/ml proteinase K in 20mM Tris-HCl, 2mM CaCl₂, pH7.5 for 7.5 minutes at 37°C (Kallioniemi et al 1994 used 0.1ug/ul) produced no improvement in hybridisation efficiency with 3 day-old slide-bound chromosome preparations, and that methanol:acetic acid (3:1) fixation of slides for one hour adversely affected signal.

3.3.2 CGH with Directly Labelled DNA

Direct labelling and detection was quickly adopted for all subsequent CGH experiments because of its convenience. Prior to clinical application, modifications to a standard protocol were briefly investigated to determine if these could make CGH simpler, faster or more reliable and therefore more readily applicable in a routine diagnostic environment. This included examining the effect on signal quality of newly available commercial products including CGH kits. Some of the modifications examined are listed in Table 3-23 and the results of these detailed below. The standard direct protocol used for comparison was essentially that of Kallioniemi et al (1994), but using 800ng rather than 200ng of each DNA per 2cm² hybridisation area in line with the prevailing drift upwards in CGH DNA concentration (by 1996, Kallioniemi et al were using 400ng).

Where the effect of a modification on hybridisation quality was significant, it could be assessed by subjective observation. An attempt was made to provide a more objective

measure of signal intensity by calculation, for each fluor, of average automatic exposure times from several cells. This was abandoned as meaningful comparisons were difficult to achieve because non-chromosomal fluorescent bodies in the vicinity of metaphases affected exposure times.

A preliminary comparison of denaturation conditions was first undertaken. This showed that formamide denaturation of 1 to 3 day-old slides at 73°C or 80°C for 3 minutes produced metaphases of broadly acceptable quality, on examination prior to CGH, with either temperature. However DAPI staining was generally paler with 80°C denaturation, more C-bands were apparent and chromosomes did appear more 'ghost-like'. A denaturation temperature of 73°C (as used by Kallioniemi et al 1994) was therefore confirmed as appropriate for 'in house' slide preparations.

3.3.3 The Effect of Modifying Elements of the CGH Procedure

i) Use of frozen metaphase preparations

Many protocols advocate providing metaphase preparations of suitable quality for CGH by preparation of large numbers of slides, testing of each batch and freezer storage, in separate containers, of small quantities of slides. This time-consuming method was investigated to determine if it produced optimal results and if an alternative approach might be more convenient.

On several occasions, as in tests 2 and 8 (Table 3-23), slides which had not been stored at -20°C were assessed against those which had been frozen. Although used by many groups to allow extended utilisation of satisfactory batches, in this study frozen slides produced poorer CGH quality (though analysis on the frozen slide might still be possible).

ii) Use of commercial target slides

The introduction of high mitotic index, well spread commercial target metaphase preparations (Vysis) promised great convenience. However these frequently appeared damaged, with many of the metaphases found to be covered with cytoplasm following processing (test 13). Analysis was sometimes possible but protracted slide scanning and imaging was required to identify suitable metaphases. After repeated complaints and trials of different batches, the manufacturer disclosed that the slides were being inappropriately frozen and thawed in transit. Despite eventual availability of correctly transported slides, quality barely improved (test 14). As reported below, increasing denaturation time to 3.5 or 4 minutes (an increase presumably required because of the slide age-Vysis even suggested 5 minutes but this caused extensive damage) could

improve the signal quality obtained from Vysis slides (tests 22, 23), but in-house, unfrozen, preparations of metaphase spreads were subsequently used where possible.

iii) Altering the fluorescent label and/or its concentration

The use of a double concentration of nucleotide-conjugated fluor in the labelling mix (tests 1 and 2) produced no improvement in signal strength.

The effect of different fluorescent labels was briefly assessed, incorporating these with the standard Gibco BRL nick translation kit (tests 4-12) or an alternative labelling system (Amersham or Vysis). Amersham fluor-X did appear to produce an enhanced green signal. This improvement was, however, variable, or only present when the fluor-X was incorporated using the Amersham labelling reagents supplied with this fluorochrome (test 3). As CY3 did not produce red signal enhancement, an alternative red fluor would have to be used, incorporated by the Gibco BRL system. This parallel use of two labelling systems was judged unwieldy and the use of fluor-X not pursued. An Amersham dUTP-FITC was later marketed separate from a labelling kit, but when incorporated by the Gibco BRL reagents this produced a weaker green signal than the NEN fluor (test 12). Signal produced by Vysis SpectrumRed label was very bright when 1ul (20uM) was used in labelling and the standard 800ng of labelled DNA hybridised, too weak when 0.5ul used in labelling and 200ng hybridised (Vysis recommend 100ng) and satisfactory when 500 or 600ng employed (tests 4, 6, 7 and 9). Acceptable signal was also obtained with 500 or 600ng of SpectrumGreen labelled DNA in 10ul hybridisation mix (test 7, 9).

Only 10uM SpectrumRed-dUTP or SpectrumGreen-dUTP was therefore used in labelling. Addition of dTTP to bring combined dUTP/dTTP concentration to 20uM (like that of each of the other dNTPs) produced no obvious benefit (test 10) and the labelled fragments were smaller. Use of a lower concentration of labelled-dUTP (10uM) than each of the other dNTPs (20uM) and topping up the concentration with dTTP is recommended by Vysis because, though label incorporation is lower, the polymerase may be more efficient.

iv) Use of the Vysis CGH nick translation reagent kit

Hybridisation quality across the slide was more variable when the Vysis CGH nick translation kit (Vysis 32-801024) was used for labelling (test 11). This system was also more expensive than the Gibco BRL kit and involved longer incubation (2-4 hours instead of 1 hour), therefore no attempt was made to optimise it.

v) Co-denaturation of probe and target DNA

Co-denaturation of probe and chromosomes using a hotplate (74.5°C, 5 minutes) was investigated, as directly-labelled chromosome paints can produce improved signal with this approach (Oncor, verbal communication). This would additionally have reduced

processing time, but though it was initially found to be very successful (test 15), the result was in fact variable as the degree of denaturation increased noticeably with only slight reduction in slide age (tests 16, 17). Using different co-denaturation temperatures or times for different ages of slide might have addressed this problem, but the approach was abandoned to avoid introducing this unnecessary complexity and as it was noted that the DAPI banding obtained following co-denaturation could be poorer.

vi) Use of commercial labelled control DNA

Commercial labelled control DNA (Vysis female and male) was tested, as it offered convenience, by halving the number of labelling reactions required, and the possibility of improved and consistent red signal quality. A higher concentration than that suggested by the manufacturer (150ng or 200ng instead of 100ng in the hybridisation mix) was required in order for the signal to be clearly visible without imaging (tests 18, 19, 20). It gave stronger, though still poor, signal than the in-house control DNA hybridised to a different area on the same Vysis slide (test 20). Using 200ng Vysis control DNA on Vysis slides denatured at 3.5 or 4 instead of 3 minutes could generate acceptable, if variable, results (tests 21, 22). However the best signal was obtained using Vysis control DNA on in-house slide preparations (denatured for 3 or 3.5 minutes, test 24) so this approach was accepted as standard.

vii) Alterations in post hybridisation washing

Reducing the post hybridisation formamide wash temperature to 43°C produced no improvement in signal intensity (tests 1 and 2).

0.4xSSC + 0.15% Tween 20 was assessed as an alternative stringent wash, in hope of improving on the background removal achieved with 50% formamide or at least maintaining this standard while using a time-saving, less hazardous, salt solution. Use at 73°C for 2 minutes resulted in partial destruction of the cell sheet on Vysis slides (test 25). Lowering the temperature to 70°C for 2 minutes produced the same result as a formamide wash, offering potential as a more convenient, safer approach (test 26). An attempt was made to reduce the excessive background present following some hybridisations by removing the coverslip and additional slide washing. A 4XT wash for 5 minutes at 37°C followed by 0.4xSSC for 1 minute did result in a slightly cleaner preparation, but, as signal was also weaker, there was no advantage in this approach.

vii) Slide pretreatment

A pre-wash in 2xSSC for 30 minutes was trialled as this could reduce the 'dirtiness' of other FISH preparations. No effect was noted on Vysis slides (test 27).

In summary, examination of procedural variations resulted in only slight modification of the protocol of Kallioniemi et al (1994,1996). In particular, commercial labelled control DNA was adopted for its convenience and consistently high quality, and the use of frozen slides was avoided where possible. When the protocol for assessing metaphase suitability was strictly followed and only the highest quality slides selected as targets, the CGH standard was usually acceptable. To improve the likelihood of a satisfactory result, CGH was generally performed on metaphase spreads from two different individuals. Alteration and improvement in analysis procedure accompanied the acquisition of Quips CGH software (Vysis). This allowed production of a set of averaged ratio profiles for the entire karyotype and interpretation became more rapid and less complex. However software-enhanced reverse DAPI banding could be poor with Quips. This was overcome by enhancing DAPI with Smartcapture software before formatting images for Quips analysis.

The most pronounced improvement in CGH signal quality followed eventual procurement of a more powerful, 100W, fluorescent light source. This additionally allowed faster exposure permitting images to be re-taken if necessary without extreme fading.

3.3.4 Initial Assessment of the CGH Protocol

CGH was performed with DNA from a neuroblastoma case with recognised abnormalities to assess the efficacy of the CGH approach.

Chromosomally Abnormal Neuroblastoma Case

Cytogenetic analysis of this case was performed on direct preparations of lymph node: 47,XY,add(1)(p31),der(3)t(1;3)(q23;p26),+9,+11-15hsr(r)[cp9]. A marrow sample from the same individual revealed a grossly abnormal karyotype with chromosome counts in the hypotetraploid range, two copies of add(1)(p31) and der(3)t(1;3)(q23;p26) in most cells plus a variable number of double minutes. FISH investigation of the lymph node sample was not possible because no cytogenetic preparation remained, though FISH was used to demonstrate N-myc amplification on the double minutes in the marrow sample. DNA was extracted from frozen lymph node tissue and CGH performed, initially with Vysis female control DNA (Fig.3-19), and interpreted with no knowledge of the cytogenetic analysis. The result was confirmed by repeat CGH using male control DNA. CGH detected substantial gain at 2p22-p24, i.e. at the N-myc locus, in this tumour DNA, which may have represented N-myc amplification in the unidentified rings. It was also able to exclude a clinically significant 17q imbalance, which would have compounded the

poor prognosis associated with N-myc amplification and loss of 1p36. Loss of 1p22-pter was demonstrated, though cytogenetic analysis had placed the add(1) breakpoint at 1p31. Gain of 1q was disclosed by CGH, but appeared to involve approximately the whole arm whereas karyotyping placed the breakpoint at 1q23. CGH also detected gain of 9p13-q12, 9q22.1-q22.2 and 9q33-qter, although visual examination actually suggested gain of an entire chromosome 9 as reported in the karyotype.

CGH did not detect the deletion of 3p26-pter from the der(3), probably due to its small size and telomeric position, but registered an apparent gain of 4q (4q28, 4q32-q34) which was not included in the cytogenetic karyotype. The 4q gain could represent the unidentified material present on the cytogenetically determined add(1)(p31) chromosome.

3.3.5 Clinical Application of CGH in Cases of Abnormal Phenotype

The technique was then applied to some examples of the type of cytogenetic problem CGH might be expected to be useful in resolving. Testing was generally repeated at least once, to confirm results and exclude the significance of slight variations. Normal male versus normal female control hybridisations were performed to assess normal ratio variation and assist threshold setting (Fig.3-20).

3.3.5.1 Testing of Patients with Apparently Balanced Constitutional Chromosomal Rearrangements but Abnormal Phenotypes

Patient KD

This case represented a familiar cytogenetic problem - apparently identical balanced karyotypes in phenotypically normal and abnormal individuals in the same kindred. One explanation is the presence of a cryptic imbalance in the affected individual(s).

Cytogenetic analysis identified that patient KD, who exhibits congenital abnormalities including dysmorphism and MR, carries an apparently balanced translocation involving the short arm of chromosome 10 and the long arm of chromosome 14

(46,XX,t(10;14)(p13;q24)), a rearrangement also present in phenotypically normal members of her family. DNA from patient KD was hybridised five times, producing analysable preparations on only three of these occasions. Preparations were always considerably poorer - mostly 'dirtier' - than control hybridisations. On electrophoresis following labelling, fragments were noted to be rather small and the DNA smear weak. Electrophoresis of unlabelled KD DNA alongside a confirmed quantity of control DNA showed far less high molecular weight DNA in the KD lane, and, indicated by the smear,

that a proportion of the KD DNA was degraded (Fig.3-21). As no other DNA sample was available from this patient, the amount of KD DNA used in labelling was increased by 50% which appeared to compensate at least in part for its poor quality, and resulted in the analysable CGH preparations.

Of the three experiments analysed, one used normal male control DNA, one Vysis male and the other Vysis female control DNA. Normal male versus female control hybridisations showed that a threshold of 0.8/1.2 was sufficient to detect loss of the entire X and gain of entire Y without introducing false positive gains and losses.

No consistent regions of gain or loss were detected in the test DNA over the three experiments at thresholds of 0.8/1.2 or 0.85/1.15. Any regions of gain or loss detected in individual experiments involved variable telomeric or heterochromatic regions (Table 3-24).

Patient GS

On conventional cytogenetic analysis, this individual, who exhibited Asperger's syndrome and mild MR, was shown to have an apparently balanced pericentric inversion 46,XY,inv(7)(p15q21.2). Separate CGH experiments with two different male and one female control were performed to exclude possible subtle segmental aneusomy at this, or another, chromosome site but revealed no consistent or significant imbalances using either Quips (Vysis) or Smartcapture 'global' analysis software (Digital Scientific).

Patient SM

Cytogenetic analysis of phenotypically abnormal patient SM had disclosed an apparently balanced *de novo* translocation involving chromosomes 5 and 6 (46,XY,t(5;6)(p11;q11)). The smaller derivative chromosome (comprising chromosomes 5 and 6 short arms) was identified as the derivative chromosome 6 and the larger as the derivative chromosome 5 by FISH with alpha satellite probes.

CGH experiments were performed as the patient's phenotype (which included profound MR, cleft lip/palate and facial dysmorphism) was inconsistent with a balanced abnormality. Reference DNAs were from two different female controls. Preliminary examination of images using global analysis showed loss in mid 1q as the only consistent imbalance. CGH interpretation of seven CGH karyotypes revealed a deletion involving 1q25-proximal q31 (.rev ish dim(1q25q31)) with a threshold of 0.8/1.2 (Fig.3-22). The limits of the 99% CI of test versus reference CGH ratio profiles were used as thresholds and did not contain 1 at the site of this apparent imbalance, supporting its significance (Weiss et al 1999 advocate this approach for identifying gains and losses). No extra gains or losses were detected with the more permissive threshold 0.85/1.15.

This result was verified by re-examination of G-banded preparations (Fig.3-22).

3.3.5.2 Testing of Patients with Apparent but Unresolvable Chromosomal Abnormality

Here CGH was applied to the characterisation of structural abnormalities which were apparent but difficult to resolve cytogenetically, and which may or may not represent clinically insignificant variations.

Patient LF

Cytogenetic analysis of LF, a patient with mild MR, seizures and multiple physical abnormalities, revealed a 46,XX,?del(21)(p) karyotype. The small chromosome 21 derivative was considered to represent a chromosome 21 with deletion of the short arm. In light of the patient's phenotype a possible deletion of 21q material was investigated. FISH with 21q21 partial chromosome paint (Biovation), the only proximal chromosome 21 probe commercially available at that time, revealed signal on both chromosomes 21. It was not possible to discern any size difference in the signals on the different chromosomes 21. As already reported, YACs 949b09 and 937e12 were also hybridised to metaphase spreads from this case. All metaphases examined showed no deletion of these sequences on either chromosome 21.

Separate CGH with two different female control DNAs (one Vysis) revealed no consistent or significant imbalance. 'Global' analysis with Smartcapture software also revealed no abnormalities.

Patient LH

Cytogenetic analysis of patient LH, who exhibited severe developmental delay and physical abnormalities including webbed neck, kyphosis and facial dysmorphism, revealed a 46,XX,v(9)(p13) karyotype. This variant 9p13 was also found in other, phenotypically normal, family members. Three CGH experiments, carried out with different male control DNAs, failed to disclose definite abnormality.

In two hybridisations (one assessed only with global analysis) which used the same in-house male control DNA but different template metaphases from different patient specimens there was regular apparent gain around the 9 centromere (at very proximal 9p and 9qh). So much that, in the hybridisation analysed with Quips software, the peak was still marked at 0.4/1.4. This effect may have been a reflection of wide variation in chromosome 9 pericentromeric repetitive sequence content, and blocking, between the in-house control and patient DNA. In three further hybridisations using other control DNA this effect was not repeated - with Vysis control DNA only a very slight gain was seen at 9cen (as well as 3cen and 14q13) with a 0.85/1.15 threshold. However, as the apparent

imbalance in the original hybridisations involved an area adjacent to the variant region of the patient's karyotype, it could not be discounted entirely.

Subsequent FISH with whole chromosome 9 paint and 9 classical satellite probe also detected no abnormality.

Patient KK

Initial cytogenetic analysis of this infant's lymphocytes revealed a 46,XY karyotype, although the quality of the preparation was inadequate for the exclusion of a small structural abnormality. Skin biopsy analysis resulted in reporting of an 'unusual' chromosome 17 which might represent normal variation. DNA was prepared from cultured fibroblasts and CGH performed, blind to the cytogenetic evaluation, using only the global analysis approach as interpretation software was not available at that time. Fifty percent of cells analysed suggested a region of loss on the short arm of chromosome 17 (Fig.3-23). This triggered FISH testing, which revealed deletion of 17p11.2 including the Smith-Magenis (D17S258) region but not Charcot-Marie-Tooth 1A (D17S122) region, karyotype: .ish del(17)(p11.2p11.2)(D17S122+,D17S258-). Miller-Dieker (D17S379 17p13.3), 17p subtelomeric (D17S2199), RARA (17q12) and 17 alpha satellite sequences (D17Z1) were all present on the deleted chromosome at their normal loci. Confirmation of these findings on lymphocyte preparations was not possible as no material had been retained and the infant died, aged 6 months. Cytogenetic analysis of parental blood disclosed no abnormality.

Patient LS

This patient had speech delay and slightly abnormal toes. Cytogenetic analysis revealed one abnormal chromosome 3 with a small amount of unidentified additional material and FISH demonstrated total 3 paint coverage of this chromosome and deletion of 3ptel. CGH was performed with male control DNA (Vysis) on metaphase preparations from two different normal individuals. Software interpretation, using the 0.8/1.2 threshold suggested to be appropriate by the normal male:female control CGH, confirmed loss at 3p telomere and showed gain at 3p24.2-p25 (Fig.3-24), karyotype: 46,XX,add(3)(p26).rev ish der(3)dup(3)(p24.2p25)enh(3p24.2p25)del(3)(3p26)dim(3p26) Slight gain was also apparent at other locations (1p36.1-pter, 19q, distal 9q and distal 22q) all of which are regions of recognised CGH variability. Hybridisations to 1p and 9q telomere FISH probes were performed to investigate the apparent imbalances at these regions, and produced normal signal patterns.

Patient JG

A 46,X,dup(X)(q26q28) chromosome constitution was found in cultured amniocytes following amniocentesis for maternal age indication. Chromosome painting confirmed that the abnormal X was comprised completely of X material, and Xq24 macrosatellite probe sequence (Oncor) was present in the usual position and not involved in the duplication. Presence of an XYqtel sequence (Cytocell) at distal Xq demonstrated that the duplication was interstitial. Both parents had a normal karyotype. At age 3.5 the child showed normal, or only very slightly delayed development.

CGH, a technique which could have refined characterisation of this rearrangement in one step, failed, and electrophoresis of unlabelled patient DNA showed the high molecular DNA band size was approximately one quarter the intensity of that from control DNA of apparently similar concentration (Fig.3-21). A smear, the usual sign of DNA degradation, was not present, but it is possible that much of the DNA was degraded to such a degree that very small fragments were produced which 'ran off' the gel. No alternative DNA sample was available and amniocyte cultures had not been stored due to contamination.

3.3.5.3 Testing of a Patient with Abnormal Phenotype but No Apparent Chromosomal Abnormality

Patient RK

Conventional cytogenetic analysis revealed no abnormality in this patient with MR and dysmorphic features. Separate CGH investigations were performed with two different female (one Vysis) and one male (Vysis) control DNAs. On interpretation, appropriate gain/loss of X and Y with male control DNA suggested 0.8/1.2 as threshold. Neither this nor a more permissive threshold, 0.85/1.15, disclosed any consistent or apparently significant imbalances. Subsequent subtelomere screening and DG/VCFS deletion testing by FISH also failed to reveal any abnormality.

TEST	WASH CONDITIONS	LABELLED NUCLEOTIDE	TEST DNA (ng)	CONTROL DNA (ng)	SLIDE TYPE	DENATUR -ATION	RESULT
STANDARD CONDITIONS							
S T A N D A R D	2x50%F 45°C	NEN Texas Red & FITC-dUTP/ Gibco NT reagents/ No added dTTP	800ng	800ng	1-4 days old/ not frozen	Separate DNA: 70°C 5min SLIDE: 73°C 3min	
1	43°C	x2 NEN Texas Red/ FITC-dUTP					not improved
2	43°C	x2 NEN Texas Red/ FITC-dUTP			frozen		worse than 1
3		Amersham fluor-X /CY3 Amersham NT reagents			frozen		green:good red:poor
4		Amersham fluor-X / 20uM Vysis SR-dUTP Gibco NT reagents					green:poor red:too bright
5		Amersham fluor-X / 10uM Vysis SR-dUTP Gibco NT reagents		500ng			green:poor red:good paler than 4
6		Amersham fluor-X / 10uM Vysis SR-dUTP Gibco NT reagents		200ng			green:poor red: pale
7		10uM Vysis SG-dUTP/ SR-dUTP Gibco NT reagents	500ng	500ng			green:OK red:OK
8		10uM Vysis SG-dUTP/ SR-dUTP Gibco NT reagents	500ng	500ng	frozen		green:OK paler than 7 red:OK
9		10uM Vysis SG-dUTP/ SR-dUTP Gibco NT reagents	600ng	600ng			green:OK red:OK as 7
10		10uM Vysis SG-dUTP/ SR-dUTP 10uM dTTP Gibco NT reagents	600ng	600ng			green:OK red:OK as 7 but gel fragments smaller
11		10uM Vysis SG-dUTP/ SR-dUTP+ 10uM dTTP Vysis NT reagents	600ng	600ng	frozen		green:varied red:varied more variable than Gibco NT
12		Amersham FITC-dUTP Gibco NT reagents			Vysis		green:poorer cytoplasm over parts of cells

Table 3-23 Modifications to the (direct labelling) CGH protocol (continued overleaf).

TEST	WASH CONDITIONS	LABELLED NUCLEOTIDE	TEST DNA (ng)	CONTROL DNA (ng)	SLIDE TYPE	DENATUR -ATION	RESULT
STANDARD CONDITIONS							
S T A N D A R D	2x50%F 45°C	NEN Texas Red & FITC-dUTP/ Gibco NT reagents/ No added dTTP	800ng	800ng	1-4 days old/ not frozen	Separate DNA: 70°C 5min SLIDE: 73°C 3min	
13					Vysis		cytoplasm over cells
14					Vysis different batch		chromosomes 'ghost-like'
15					4 days old	co-denature 74.5°C 5min	green: better banding poorer red: better
16					3 days old	co-denature 74.5°C 5min	green: OK red: poorer over-denatured
17					1 day old	co-denature 74.5°C 5min	green: OK red: poorer over-denatured
18				100ng Vysis SR control DNA	Vysis		green: OK red: invisible
19				150ng Vysis SR control DNA	Vysis		green: poor red: poor chromosomes have 'holes'
20				200ng Vysis SR control DNA	Vysis		green: poor red: poor better than in- house control DNA
21				200ng Vysis SR control DNA	Vysis	4min	green: OK red: OK better than in- house control DNA
22				200ng Vysis SR control DNA	Vysis	3.5min	signal good cells over- denatured
23					Vysis	4min	green: OK red: OK varies (typical Vysis slide)
24				200ng Vysis SR control DNA		3.5min	green: good red: good
25	0.4xSSC+ Tween20 73°C 2min				Vysis		damaged cell sheet - no analysis
26	0.4xSSC+ Tween20 70°C 2min				Vysis		as 50%F but slide quality poor
27	pre-wash 2xSSC 37°C 30min				Vysis		particulate, holes, uneven (typical Vysis slide)

Table 3-23 Modifications to the (direct labelling) CGH protocol (continued).

Where no details are entered, the condition was standard, as described in the first row. The standard conditions used were broadly those of Kallioniemi et al (1994), but using 800ng of labelled control and test DNA.

50%F: 50% formamide in 2xSSC; min: minute; SR: SpectrumRed; SG: SpectrumGreen; Vysis SR control DNA: Vysis normal control DNA labelled with Spectrum Red; NT: Nick Translation.

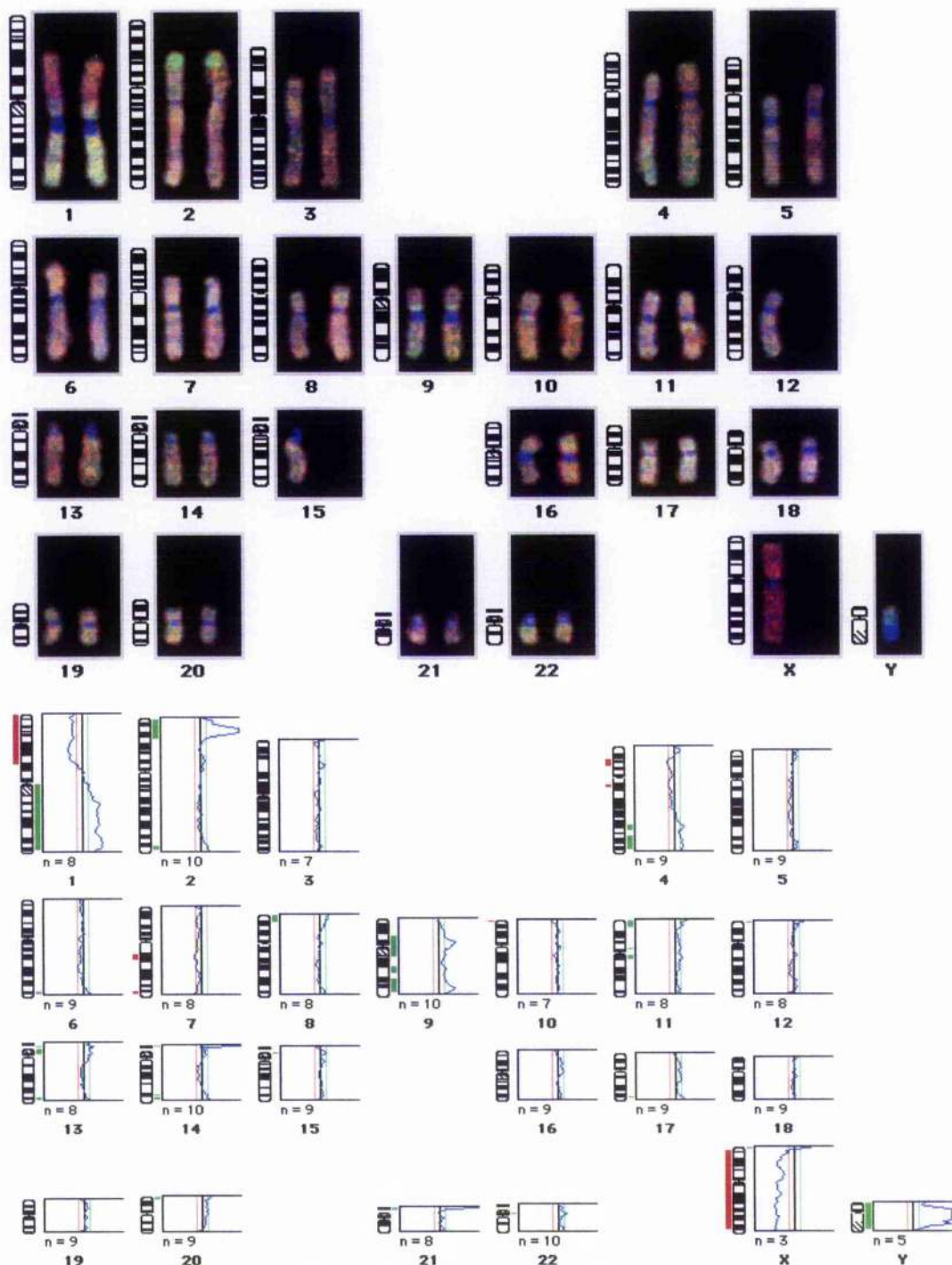


Fig.3-19 Karyotype (above) and set of averaged ratio profiles (below) following CGH with lymph node DNA from a patient with neuroblastoma. Each profile is presented to the side of the relevant chromosomal ideogram. A central line reflects the balanced state with upper and lower thresholds respectively represented by lines left and right of the central line. The test DNA (labelled green) is male, and female DNA was used as reference. CGH detects substantial gain at 2p22-p24 (green:red ratio profile peak and green bar to the right of the chromosome 2 ideogram), loss of 1p22-pter (ratio profile trough and red bar to the left of the chromosome 1 ideogram), and gain of 1q, 9p13-q12, 9q22.1-q22.2 and 9q33-qter (visual examination suggested gain of an entire chromosome 9). An apparent gain of 4q (4q28, 4q32-q34), which was not included in the cytogenetic karyotype, was also detected.

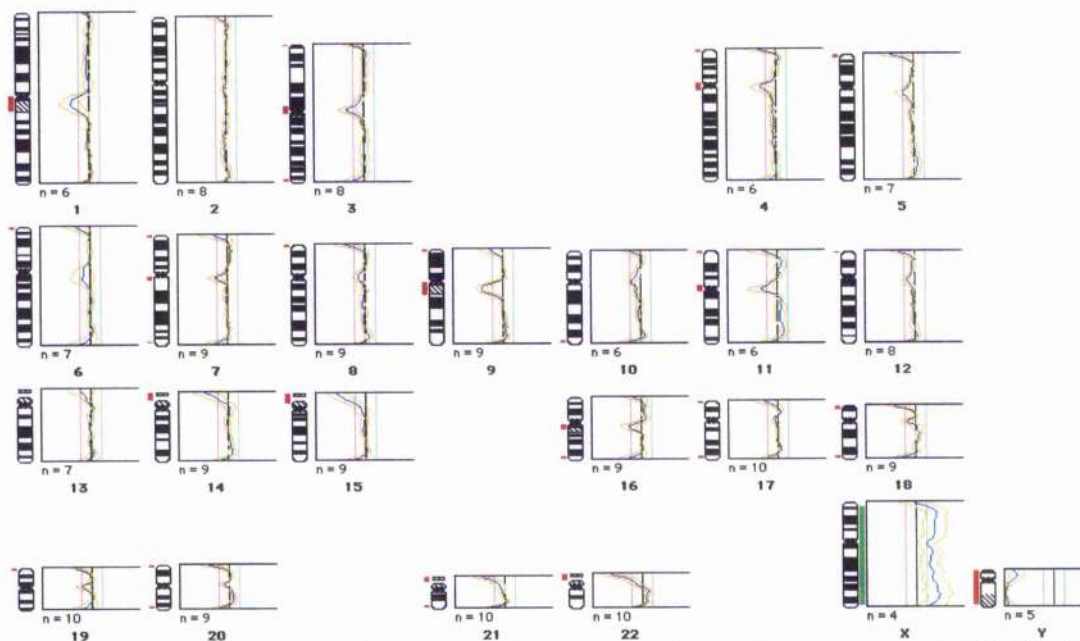


Fig.3-20 Averaged set of ratio profiles from 5 metaphases following control CGH with normal female test and normal male reference DNA.

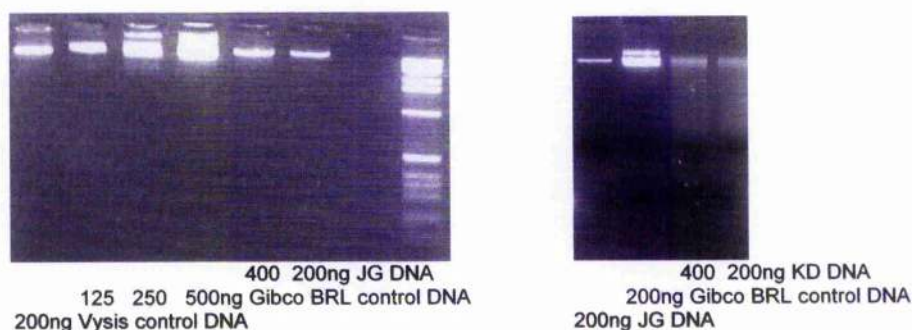


Fig.3-21 Agarose gel electrophoresis of unlabelled JG, KD, Gibco BRL plasmid and Vysis human control DNA. This demonstrated far less high molecular weight DNA in patient JG samples which had been determined to contain 200ng and 400ng by optical density readings than in the equivalent concentrations of control DNA. The unlabelled DNA from patient KD was shown to be degraded (by the presence of a smear below the high molecular weight band of DNA). JG DNA failed to produce CGH results of analysable quality and KD CGH had to be repeated several times.

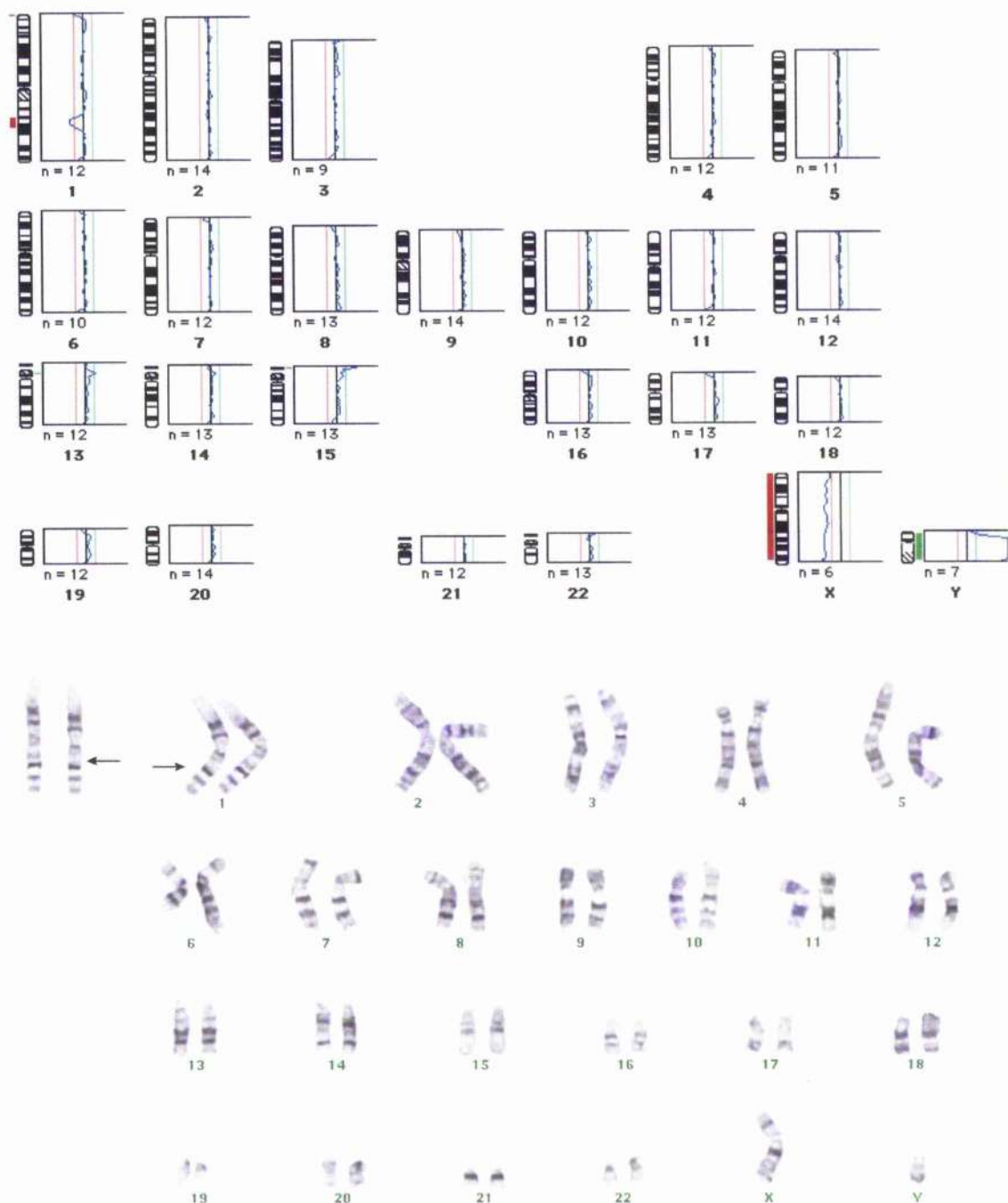


Fig.3-22 CGH to DNA from an individual (SM) with an apparently balanced t(5;6) translocation.

Above: A set of averaged ratio profiles from 7 metaphases. A variation, outwith the threshold level, in the green:red ratio at 1q25-proximal 1q31 (shown as a trough in the ratio profile and as a red bar to the left of the chromosome 1 ideogram) indicates deletion at this locus.

Below: Following CGH, re-examination of the banded karyotype clearly demonstrated this deletion (arrowed).

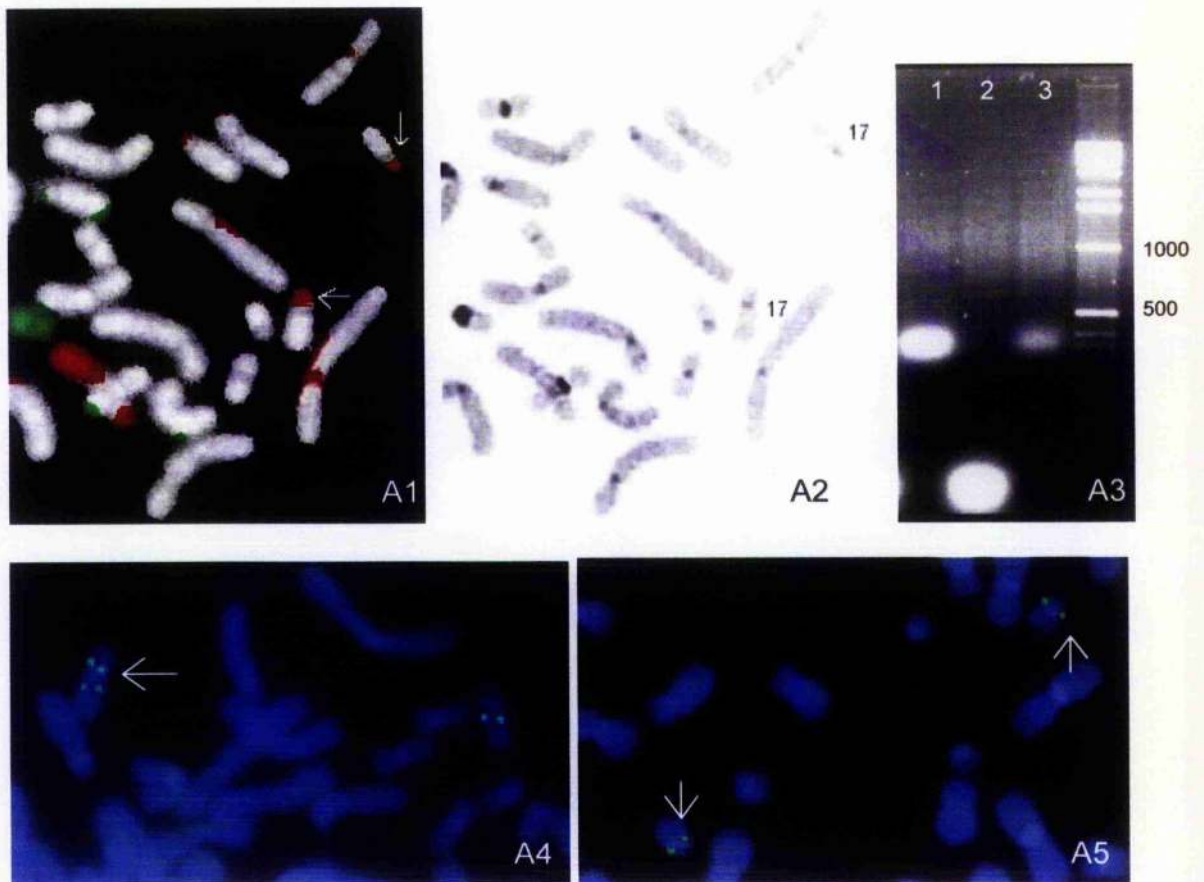


Fig.3-23 CGH and FISH analysis of patient KK.

A1: 'Global' analysis following CGH showing loss of test DNA (red areas) on the short arms of the chromosomes 17. The test DNA (green label) is from a male and the reference DNA (red label) from a female, hence chromosome X also shows red and the Y green. Other red or green areas were not consistently located and tended to be at centromeric or telomeric regions.

A2: Software-enhanced reversed DAPI banding assists chromosome identification.

A3: Agarose gel lanes 1 and 2 show control DNA and KK DNA following labelling. Smears show fragments of >500bp. Bright spots are unincorporated fluorescent nucleotides.

A4: FISH to Smith-Magenis syndrome (SMS) probe (Vysis) confirms the presence of a deletion at 17p11.2 involving this locus. The SMS signal on the undeleted chromosome 17 is arrowed. The other signal on both chromosomes 17 is control probe RARA, 17q12.

A5: FISH to commercial Charcot-Marie-Tooth (CMT) probe shows that the 17p11.2 deletion does not involve the CMT locus.

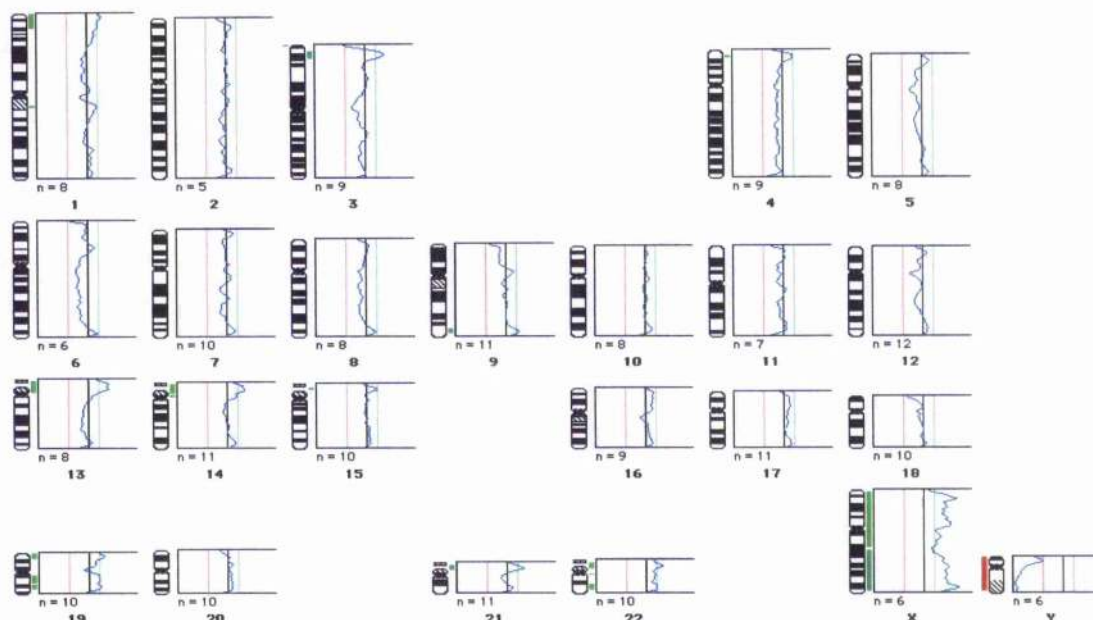


Fig.3-24 Averaged set of ratio profiles from 6 metaphases following CGH with female patient LS DNA. Reference DNA is male. Deletion at 3pter and duplication involving 3p24.2-p25 is indicated. The possible imbalances at 1pter and 9qter were excluded by FISH testing.

TEST	CONTROL DNA	THRESHOLD 1.2/0.8	THRESHOLD 1.15/0.85
KD	FEMALE (Vysis)	NO GAINS/LOSSES	GAIN (very slight): 7pter
	MALE (Vysis)	GAIN: 3pter,9pter,12pter,9qh	GAIN: as 1.2/0.8 plus 14qter,4qter,17qter,18qter
	MALE	GAIN (very slight): 5qter,8qter,2qter,2pter LOSS: 19p	NOT USED
GS	FEMALE (Vysis)	GAIN: 2q31(slight), 3q23,12q21(very slight) LOSS: 9q,19q	NOT USED
	MALE (Vysis)	GAIN: proximal Xq, proximal Xq21	NOT USED
	MALE (FITC)	NO GAINS/LOSSES	NOT USED
LF	FEMALE	LOSS (slight): parts of 4q, terminal 4q,5q,6p,6q, 7p,8p,11q,12p,18p,Xq	NOT USED
	FEMALE (Vysis)	NO GAINS/LOSSES	NOT USED
LH	MALE	GAIN: 9qh	GAIN: 9qh/cen, 9q34 (slight)
	MALE (Vysis)	NO GAINS/LOSSES	GAIN (very slight): 9cen,3cen,14q13
RK	FEMALE	NO GAINS/LOSSES	NO GAINS/LOSSES
	FEMALE (Vysis)	NO GAINS/LOSSES	NO GAINS/LOSSES
	MALE (Vysis)	NO GAINS/LOSSES	NO GAINS/LOSSES

Table 3-24 Regions of fluorescence ratio fluctuation in patients with no consistently detectable genomic imbalance on CGH.

NAME	LOCUS	SEQUENCE (5' TO 3')	SIZE (bp)	ANNEALING TEMPERATURE/ RECOMMENDED CONCENTRATION IN 50ul	REFERENCE
E570 +strand	D3Z1	TCTGCAAGTGGATATTTAAA	>1000	45-60°C 50-250ng	Gosden and Lawson (1994)
E574 -strand	D3Z1	TGAGTTGAACACACACGTAC		45-60°C 50-250ng	Gosden and Lawson (1994)
FactorIX X1	Xq27	ACCTTATGGGACCACTGTGG	370	58°C 200pmol	Cinti et al (1993)
FactorIX X2	Xq27	ATATTTCTCCTTCCTCCCTC		58°C 200pmol	Cinti et al (1993)
RBM1 forward	Yq11	ATGCACTTCAGAGATACGG	800	PCR:58°C 50pmol	Vogt et al (1996)
RBM1 reverse	Yq11	CCTCTCTCCACAAAACCAACA		PCR 58°C 50pmol	Vogt et al (1996)
DAZ forward	Yq11	GGAAGCTGCTTTGGTAGATAC	1300	PCR 58°C 50pmol	Vogt et al (1996)
DAZ reverse	Yq11	TAGGTTTCAGTGTTCGATTCC G		PCR 58°C 50pmol	Vogt et al (1996)
13A	13 alpha satellite	TGATGTGTGTACCCAGCT		60°C 100pmol	Pellestor et al (1995)
21A	21 alpha satellite	TGATGTGTGTACCCAGCC		61°C 100pmol	Pellestor et al (1995)
267	human satellite III	AATGGGATGGAGTGGGAATCAAC CCCAATGGAATCG		60°C 50-500ug	Gosden and Hanratty (1993)
435	human β satellite	AGTGCAGAGATATGTCACAATG CCCC		60°C/ 50-500ug	Gosden and Hanratty (1993)
DMD exon 6 forward	Dystrophin Xp21	CCACATGTAGGTCAAAAATGTA ATGAA	202	60°C 25pmol	Abbs et al (1991)
exon 6 reverse	Dystrophin Xp21	GTCTCAGTAATCTTCTTACCTAT GACTATGG		60°C 25pmol	Abbs et al (1991)
DMD exon 51 forward	Dystrophin Xp21	GAAATTGGCTCTTTAGCTTGTG TTTC	388	60°C 25pmol	Abbs et al (1991)
exon 51 reverse	Dystrophin Xp21	GGAGAGTAAAGTGATTGGTGGA AAATC		60°C 25pmol	Abbs et al (1991)

Table 3-25 Oligonucleotide primers used in PRINS studies.

3.4 Primed *In Situ* Labelling

These experiments principally examined means of improving penetrance to target sites (by slide pretreatment), increasing amplification efficiency (with alternative DNA polymerases and reaction conditions), and augmenting signal size and hence visibility (by cycling and TSA), in order to optimise potential for achieving detectable PRINS labelling of unique chromosomal sequences. Primer sequences used are listed in Table 3-25.

Commercial Repetitive Target Sequence Primers and Kits

Initial familiarisation with this novel process was acquired through use of the PRINS Reaction Set with a human chromosome 12 alpha satellite primer (Boehringer Mannheim). The reaction mix and recommended denaturation and annealing/extension conditions used were as test 1, Table 3-28. Satisfactory signal and minimal background was obtained using fixed-temperature hotplates or the surface of tube thermal cycler blocks for denaturation. Interphase scoring of a cytogenetic preparation from an individual with suspected B-cell CLL was subsequently performed (Table 3-26), clearly identifying a raised proportion of nuclei with 3 copies of 12 alpha satellite (60%) compared to a normal control preparation (1%).

INTERPHASE NUCLEI	NUMBER OF CHROMOSOME 12-SPECIFIC SIGNALS SCORED					
	0	1	2	3	4	5
patient	0	1	23	60	14	2
control	0	2	95	1	2	0

Table 3-26 Interphase scoring following PRINS with the PRINS Reaction Set and human chromosome 12 alpha satellite primer (Boehringer Mannheim).

PRINS with Chromosome 12 Print Kit (Advanced Biotechnologies)

During the course of later PRINS work, another commercial PRINS system, Chromosome Print Kits (Advanced Biotechnologies), became available. Limited assessment of the chromosome 12 Print oligonucleotide was carried out using both the kit procedure and reagents and alternatives to these (Table 3-27). All tests used 1 day-old slides and the recommended PCR conditions for these primers. An OmniGene flatbed (Hybaid, Fig.3-31) was used for denaturation. The recommended label, biotin-16-dUTP, was visualised only weakly with one FITC layer (test 1). Subsequent tests were therefore performed with DIG-11-dUTP label and two detection layers (tests 2-4), and all exhibited stronger signal. Identical reagents produced stronger signal when two layers of FITC were used instead of one (tests 1 and 3).

Using DIG labelling mix (Boehringer Mannheim) and AmpliTaq /S (Taq /S, tests 2 and 4), a 30% increase in enzyme (test 4) produced considerable signal increase (64% rather than 30% of 'normal' nuclei with two signals) but also increased background speckling. All test results compared poorly with those obtained with the Boehringer Mannheim primers and PRINS kit (Table 3-26), where 95% of normal nuclei showed two signals.

Chromosome 3 Alpha Satellite (D3Z1) - Specific Primers

PRINS with in-house repetitive sequence primers was investigated in order to develop experience in the preparation of PCR primers and assess the effect of altering PRINS reaction conditions. A further aim was to identify a combination of reaction components which would produce weak but reliable signals with a repetitive sequence primer, allowing its eventual use as a control when assessing the effect of different reaction conditions (including cycling) on PRINS with unique target sequence primers. A series of experiments (chronologically described in Table 3-28) was therefore performed, mostly on 1 to 3 day-old slides, with chromosome 3 alpha satellite (D3Z1)-specific primers (Gosden and Lawson 1994).

Initial PRINS with 50pmol of each primer in 30ul mix, Boehringer kit reagents and recommended denaturation and amplification/extension temperatures produced strong specific signal but also heavy background signal (test 1).

Subsequent reactions were performed on the newly-acquired OmniGene thermal cycler with flatbed slide module. On investigating the effect of altered enzyme, extension at 72°C (test 6), and varying dNTP, MgCl₂ and label concentration (tests 2-5, 11-13), satisfactory non-cycling results (signal on 100% of metaphase target sites with low background) were obtained using Taq /S, and DIG-11-dUTP (100uM), 100uM dTTP and 100 or 200uM dATP, dCTP, dGTP (tests 4, 5, Fig.3-25), with no extension step. Use of separate label and dNTP preparations was preferred, allowing independent alteration of their concentrations, but commercial label mix had been assessed because of its convenience and its use by Cinti et al (1993).

Cycling PRINS was attempted using test 4 reaction components. Extension (here at 65°C) was used in accordance with Cinti et al (1993) and to improve specificity (tests 7, 8, Fig.3-25). This with (as in Cinti et al 1993) or without formamide slide denaturation produced signal but also haze-like FITC background.

Tests 14 and 15 involved initial assessment of a cheaper and reputedly very effective PCR enzyme, Dynazyme, both with its supplied buffer and, as prolonged exposure to detergent during any cycling might cause chromosome damage, a detergent-free buffer (Gibco BRL) to which a low concentration of Triton X-100 could be added. Signal was

produced, though accompanied by a high level of background. This might have been due to reduced dTTP (as in tests 12 and 13), but prompted solution PCR to further compare amplification with this enzyme against that with Taq *IS* (see below and Table 3-29). Following this, PRINS tests 16-20 used similar components to D3Z1 solution PCR tests 3 and 5 and employed a higher annealing temperature than previous D3Z1 PRINS to attempt background reduction. The Dynazyme tests (16, 17) also used less label. One detection layer was adequate for specific signal visualisation with Dynazyme (tests 16, 17) or Taq *IS* (18, 19), but did not eliminate background. Background was reduced with Taq *IS* on lowering denaturation and increasing annealing/extension temperatures (test 20). Using Dynazyme and reduced label (33uM) with annealing temperatures of 66°C or 68°C produced, as sought, reliable, specific, signal which was not overly strong (tests 21, 22).

The effect of T4 ligase pretreatment on cycling PRINS was then investigated on 3 and 10 day-old slides, using test 22 reaction conditions (tests 24-27, Fig.3-25). Signal (and incidentally no background) was only obtained on the 3 day-old slides which had been treated with ligase (test 27), but, as in test 7, a 'haze' could be present around metaphases.

The potential benefit to cycling PRINS of, in addition to ligase, proteinase K pretreatment as employed in CGH (0.2ug/ml, 7.5min at 37°C) was initially assessed in non-cycled preparations. Even with reduced primer, reliable signal was still obtained on a control slide treated with only ligase (test 28). However, the proteinase K treatment used resulted in complete destruction of metaphases (test 29), suggesting that preparations for PRINS must require gentler pretreatment than those denatured under the milder conditions (lower temperature/high formamide) employed in FISH and CGH.

The newly reported Tyramide Signal Amplification system (TSA, Dupont, Raap et al 1995) was tried with PRINS as an alternative to cycling, because the latter consistently resulted in poor post-PRINS metaphase quality. Reactants and conditions were as used in tests 20 and 21. On the recommendation of the TSA system manufacturer, stop buffer was omitted in case it inhibited TSA enzymes. Many, bright, signals were produced (tests 30, 31). Simultaneously, TSA of the FISH signal from DIG-labelled H1012 probe was performed following one FITC layer (Fig.3-26). This produced stronger specific 22q11 signals than on a control slide and bright signals elsewhere, confirming the efficacy of TSA but further demonstrating the reduction in specificity it could produce.

Solution PCR with D3Z1 Primers

While recognising the results may not reflect those obtained in PRINS, PCR was performed in solution to provide some assessment of the effect on amplification of altered enzyme and reduced $MgCl_2$ and primer concentration.

As initial tests showed DIG-labelled DNA failed to migrate on electrophoresis only unmodified nucleotides were used. Reducing primer (to 25 from 50pmoles/30ul), with Taq /S or Dynazyme, most effectively reduced product while improving specificity (Table 3-29, Fig.3-27A). Reagent combinations 3 and 5 appeared to be equal candidates for a reaction mix which might produce good results on cycling PRINS.

PRINS with Factor IX Primers

Both PRINS and, because Cinti et al (1993) had described Factor IX signal as 'barely detectable with conventional fluorescence microscopy', cycling PRINS was attempted with these primers.

The reaction mix (tests 1, 2, Table 3-30) was essentially as reported but with Taq /S. One day-old slides were used as it had been shown (D3Z1 test 25) that slide age could affect amplification efficiency. Denaturation involved an OmniGene flatbed (effectively essential in cycling) whereas Cinti et al (1993) had used formamide. Detection had two FITC layers to assist amplification visualisation. These tests failed to produce specific signal, which was expected as the reaction mix, like that reported by Cinti, contained no $MgCl_2$.

Subsequent, non-cycling PRINS with 10 day-old slides and formamide denaturation (as Cinti et al 1993) was performed both with and without $MgCl_2$. Signal was obtained with $MgCl_2$ in the control (D3Z1 test 9), but mainly speckling on the Factor IX-primed slide (Table 3-30 test 4). Possible signal was visualised on the distal long arm of just one chromosome X in the preparation. No signal was produced in the absence of $MgCl_2$ with either Factor IX (test 3) or control primers (D3Z1 test 10).

Solution PCR with Factor IX Primers

Solution PCR was performed to check the primers were functioning and to assess the suitability of different buffer/enzyme and primer concentrations and annealing temperatures (Table 3-31, Fig.3-27 C, E).

Though ammonia traces following primer evaporation can affect amplification (Flowgen, personal communication), evaporated and precipitated primers (Table 3-31 tests 1, 2) both produced nonspecific bands demonstrating that evaporation had not been the cause of nonspecificity here. Controls with evaporated DMD exon 6 primers did amplify specifically. Reducing primer and raising annealing temperature (tests 4, 5) failed to

improve specificity. Use of Taq /S (test 6) did give some improvement, though increasing $MgCl_2$ (to the 4.5mM recommended for *in situ* use, test 7) again produced nonspecific, though stronger, bands (this pattern of increased band strength but loss of specificity was mirrored in controls). Further reduction of primer (to 20pmol) and increased annealing temperature (63°C) with Dynazyme could result in a strong, specific band (~370bp, test 8).

In summary, Factor IX-primed solution PCR worked best with Dynazyme and at lower primer concentration and higher annealing temperature than cited by Cinti et al (1993) for PRINS with these primers. However successful, specific, amplification might not be consistently reproducible (test 9).

PRINS with Dystrophin Primers

PRINS with these alternative unique sequence primers (Abbs et al 1991) was attempted as they were known to work well in PCR in this department and because DMD/BMD multiplex primers represented a readily available set of nested sequences. PRINS with several primers for the same gene, an initial intention of this study, seemed one way to approach signal amplification. Individual primers, exon 51 oligonucleotides, were investigated first, with repetitive primers 267 and 435 as positive controls, during assessment of a loaned Perkin Elmer *in situ* PCR system. Using the same cycling conditions as in solution PCR, no exon 51 signal was obtained (Table 3-32 tests 1, 2). At 30 cycles standard Taq polymerase (Boehringer Mannheim) produced extensive damage, confirming the importance of using reagents with minimal detergent in cycling PRINS. Subsequent tests (3-6) involved exon 6 primers and used Taq /S, two FITC layers and raised concentrations of primer, $MgCl_2$, label and dNTPs, to maximise the likelihood of obtaining signal. Both formamide and OmniGene denaturation were used (tests 3, 5). Enzyme was omitted in tests 4 and 6 to assess the degree of background caused by the detection system, which was minimal. Substantial signal, all of it nonspecific, was only obtained in the presence of enzyme (Fig.3-28).

Solution PCR with Dystrophin Exon 6 Primers

The dystrophin exon 6 primer set was used as a (single-copy target) positive control in solution PCR when testing Factor IX primers and the effect of alternative buffer/enzyme combinations, and enzyme, primer and $MgCl_2$ concentrations (Table 3-33, Fig.3-27 C, D, E). Equivalent, specific, amplification was achieved using Dynazyme or Taq /S and 1.5mM $MgCl_2$ (tests 1-4). Doubling the primer concentration produced no apparent increase in yield (tests 7, 8). Raising $MgCl_2$ to 4.5mM could produce slightly increased

amplification but also some nonspecific product (test 5). Test 6 was run to confirm that detergent omission resulted in amplification failure (as in DAZ tests 5 and 6, Table 3-34).

Amplification of Y-specific Sequences

DAZ (Reijo et al 1995, Vogt et al 1996) and RBM1 (RNA-Binding Motif, formerly known as Y chromosome RNA Recognition Motif, YRRM, Ma et al 1993) sequences are candidate genes for male germ cell differentiation. These gene families map to the Azoospermia Factor (AZF) region of Yq11.2 that controls spermatogenesis. Primers for these sequences were being used in this department (Thesis of Mohammed FM, 1999) when their behaviour in PRINS was briefly investigated.

Solution PCR with DAZ Primers

The DAZ transcription unit spans about 42kb, including nine tandem repeats of a 2.4kb unit (Saxena et al 1996). Published DAZ oligonucleotides have a large amplification product (1300 base pairs, Vogt et al 1996), which suggested that they could be useful as PRINS primers.

PCR with these primers was firstly investigated in solution, to identify the optimal constitution of the reaction mix (Table 3-34, Fig.3-27 D, E). Even with 4.5mM MgCl₂, Taq /S produced a weaker band than 2.5U of standard AmpliTaq (tests 1,2), though increasing Taq /S did give acceptable amplification (test 3). Dynazyme with Dynazyme buffer produced a strong band on electrophoresis (test 4). As cycling PRINS would require minimal detergent, buffer minus detergent was tested with AmpliTaq and Dynazyme (tests 5, 6). In the absence of added detergent the PCR failed, but a Dynazyme mix with 0.01-0.02% Triton X-100 amplified well (tests 7,8).

Cycling PRINS with DAZ Primers

DAZ solution PCR mix 3 was selected as the basis for a DAZ cycling PRINS reaction mix as it produced an extremely specific, but not overly strong, band. Both 30 and 100pmoles of each DAZ primer were used in cycling PRINS, but no signal, only speckle background, was produced, with metaphase damage (Table 3-35 tests 1, 2). TSA of a separate 30pmole reaction produced no speckling but spurious, bright fluorescence on a few chromosomes (test 3).

Solution PCR with RBM1/YRRM Primers

RBM1/YRRM sequences, expressed specifically in the testis, may map to Yp (Vogt et al 1996) as well as Yq11 (Ma et al 1993, Vogt et al 1996). They were reported to occur as

multi-copy clusters, which suggested PRINS with primers for these sequences might be successful.

Again solution PCR was used first to assess different reaction conditions with these primers. The effect of Taq /S and Dynazyme with low detergent was compared with amplification using established RBM1/YRRM PCR conditions (Table 3-36, Fig.3-27B). The specific 800bp electrophoresis band was produced by all reaction mixes used.

Cycling PRINS with RBM1/YRRM Primers

PRINS was initially performed using Taq /S with 4.5mM MgCl₂ (Table 3-37 test 1) as higher MgCl₂ concentrations are recommended for PRINS and the equivalent PCR mix (Table 3-36 test 3) had produced very specific amplification. It was thought TSA might work optimally in the absence of nonspecific product. In retrospect the primer concentration (30pmol/50ul) might have been too low, but increasing DAZ primer to 100pmol had given no improvement. Cycling (30 cycles) damaged metaphases, and TSA following cycling (test 2) gave very bright, nonspecific signal.

Biotin PRINS labelling with fewer cycles (10) was also attempted (tests 3, 4), in an effort to reduce damage. It was hoped the amplification effect of the three layers used in detecting biotin with two layers of FITC might compensate for fewer cycles. No convincing signal was produced, but speckles were present on chromosome Y in a few cells (test 3). TSA (test 4) failed to amplify signal and caused a 'clouding' effect.

As chromosome damage during or prior to heat treatment might have been the cause of failure, denaturation, annealing and extension temperatures were reduced slightly and the effect of using older (10 day) slides with ligase pretreatment assessed (tests 5, 6).

Reaction mixes were based on PCR test 6. No improvement was conferred, with or without ligase (as in D3Z1 tests 24, 25), and only nonspecific speckle produced.

PRINS with Primers for Human Satellite III (267) and β Satellite (435) DNA

Experiments with these primers investigated their potential as a more objective means (than just signal intensity) of monitoring the effect of altering PRINS reaction conditions and of amplification.

Oligonucleotides 267 and 435, for human satellite III and β satellite DNA respectively, had been used to demonstrate the increased sensitivity of cycling PRINS (Gosden and Hanratty 1993). Satellite III DNA is present at the centromeric regions of chromosome 9, the acrocentric chromosomes and chromosome 16 (in decreasing order of sequence copy number). Human β satellite sequences occur in relatively few copies on a number of chromosomes and in higher copy number on some acrocentric chromosomes and

chromosome 1. It was reported that cycling augmented not just signal strength but the number of chromosomes exhibiting signal, the sites with fewer sequence copies becoming gradually detectable as cycle number increased. With a single PRINS cycle, 267 primer gave signal only on chromosome 9. After five, signal was also visible on 16 and all acrocentric chromosomes. Primer 435 labelled six sites after a single cycle: chromosomes 1, one pair of acrocentric chromosomes and, weakly, chromosomes 9. Thirty cycles revealed eighteen sites, including all acrocentric chromosomes.

A collection of forty five tests were performed (Tables 3-38, 3-39). These attempted to reproduce the published results then use the model obtained to gauge the success of cycling and amplification experiments.

Initial PRINS was essentially according to Gosden and Hanratty (1993), but replaced AmpliTaq with Dynazyme and low detergent buffer to minimise cell damage during any cycling. Signal excess with this mix necessitated tests 2-4 (Tables 3-38, 3-39, Fig.3-29A), designed to reduce the signal number in one cycle to that reported (Gosden and Hanratty 1993). Rough equivalence to the published signal pattern, convincing signal only on chromosomes 9, was achieved in 267 test 4. Efforts then focussed on 267 as primer 435 PRINS appeared more difficult to optimise.

Since a promising result had been obtained in 267 test 4, similar conditions were employed in single and five-cycle PRINS but using ligase pretreatment and either increased dTTP or less primer to attempt to remove small excess signals (tests 6-10). However use of 1 day-old slides for these tests, as would be preferred in rapid diagnostic work, actually resulted in an increase in signals compared with test 4.

Increasing the annealing temperature, with no ligase, produced no reduction of signal in 1 day-old slides on single cycle PRINS, instead giving increased background speckling (tests 17-20). Neither increasing dTTP nor decreasing primer in these tests removed this background, though signal strength and number was slightly reduced. Test 28 also had raised annealing temperature and it too showed excessive speckling and weakened specific signal.

Reverting to 63°C annealing, a further reduction in excess signal (including most of the weak signal on acrocentrics as seen in test 4) was as expected achieved by use of 6 day-old slides and reduced concentrations of labelled and unlabelled nucleotides and primer (tests 21, 22).

On 1 and 3 day-old slides with the same dNTP mix and annealing temperature as test 22, but less primer, the signal number was again higher than in older slides (tests 24, 26). Test 25 (1 day-old) also gave more signal than test 23 (6 day-old slide). Further primer

reduction (3pmoles, test 27) produced fairly strong signals only on chromosomes 9 (Fig.3-29B).

PRINS with five cycles, tests 29 and 30, was then performed using the same conditions as tests 26 and 27, including 63°C annealing. This produced 'haze' and 1-2 signals per cell which were faint, especially with only 3pmoles of primer (test 30). However cycling using 65°C annealing could paradoxically produce slight signal increase (test 31 Fig.3-29C, compared to test 29).

On 1 day-old, ligase treated slides, annealing at 65°C for the first cycle only, then at 63°C (test 37) in an attempt to reduce damage failed to improve this result. Ten, rather than five, cycles with test 37 conditions (test 38) gave no signal at all, though raising primer to 20pmoles produced six signals (test 39).

TSA without cycling was attempted (tests 13-16) with 1:100 anti-DIG antibody, as a 1:50 dilution had produced excess amplification in D3Z1 tests 30 and 31 (Table 3-28).

However TSA (tests 13, 14, 15) produced green 'haze' and no improvement in metaphase signal strength or number compared with equivalent non-amplified preparations (tests 6,7, 8).

Test 34 (Fig.3-29D), involving TSA following single cycle PRINS with only 5pmoles of primer, no ligase, reduced dNTP concentrations and 65°C annealing (as in cycling test 31) again produced green 'haze' but possibly larger, more diffuse signals than the non-amplified control (test 33). Contrary to D3Z1 findings, there was no evidence in 267 tests (e.g. 35, 36) that ligase treatment could reduce nonspecific background signal.

Fixation in 3:1 methanol:acetic acid followed by 37°C incubation in 2xSSC had been introduced, initially for newer slides, as this could produce slightly 'cleaner' FISH preparations. However fixation appeared to increase penetrance (tests 6 and 7, 9 and 11, 10 and 12, 14 and 13, 16 and 15). As this was not the aim in these particular experiments, no fixation, only 2xSSC incubation, was used in later tests. Probably the best non-cycling (test 27) and cycling (test 31) results were achieved with this pretreatment - though this may have been due to other condition alterations described above.

Mild proteinase K slide pretreatment (0.2ug/ml, 3 minutes at 37°C) instead of fix/2xSSC (test 5), appeared to improve the preparation. Not, as expected, by increasing penetrance, but by reducing 'speckle' background and rendering the specific signal on chromosome 9 more distinct, with other signals diminished.

PRINS with Chromosome 13 and 21-specific Primers (13A and 21A)

An attempt was made to replicate the specific results obtained by Pellestor et al (1994,

1995a,b) with chromosome 13 and 21 alpha satellite primers. DIG label was used for this study as this produced stronger signal when one detection layer was used (Pellestor et al 1995b). The Boehringer DIG labelling mix has less dNTP (100uM instead of 200uM), more labelled dUTP (35uM rather than 20uM) and dTTP (65uM rather than 20uM) than used by these authors. One day, rather than the recommended 3 to 10 day-old, slides were used because the main use for this PRINS would be in rapid testing, e.g. in PND. With 13A primers annealed at 60°C, only 14 of a possible 38 signals were present on chromosome 13 in 19 metaphases even with two, rather than one, detection layers to optimise signal visualisation. Three of these cells also showed one chromosome 21 signal and one had two chromosome 21 signals. PRINS with 21A primers and annealing at 61°C gave strong chromosome 21 signal but a lot of speckle background. Twelve of a possible 14 chromosome 21 signals were present in seven metaphases, four of which also showed in total 6 signals on chromosome 13 and two of which also showed 3 signals on other chromosomes (Fig.3-30).

This work was abandoned as it continued to give nonspecific results and because of concerns regarding the robustness and reliability of this approach in a clinical setting (just 1°C variation in annealing temperature produces both chromosome 21 and 13 signal in all cells).

Additionally Pellestor et al (1994, 1995a,b) reported both that the 13A primer is polymorphic, producing no signal on one 13 homologue in one of ten individuals tested, and that 'background staining can sometimes be observed' with 21A.

Overall Summary of PRINS Investigations

Although optimal PCR conditions might not correspond to those in PRINS, solution PCR was used to provide some assessment of primer efficacy and of the effect of modifying reaction mix constituents and reaction conditions. However, though optimisation of solution PCR was generally achieved with the unique primers tested, PRINS was not. Convincing PRINS signal was only obtained with repetitive sequence primers (D3Z1, 267 and 435, 13A, 21A), where amplification occurs over a longer DNA stretch so is more easily visualised. The signal augmentation effects of cycling PRINS and TSA were assessed with these and with unique sequence primers.

Use of standard Taq polymerase produced extreme damage on cycled PRINS preparations. Taq *IS*, specially developed for *in situ* PCR, generally rendered cycling less destructive (D3Z1 tests 7, 8, Factor IX test 2, dystrophin exon 6 tests 3, 5), though damage could still occur (e.g. DAZ tests 1, 2, RBM1 test 1). An alternative, relatively inexpensive, enzyme not previously reported in PRINS (Dynazyme) was shown, with low

detergent, to produce satisfactory non-cycling PRINS results with D3Z1 (test 21) and 267 (test 27) primers. However, as with Taq /S, significant amplification or total prevention of cycling damage was never achieved.

In some experiments (267 tests 37, 38) increased cycle number resulted in loss of signal, indicating diffusion may have been occurring. The only cycling PRINS which allowed possible visualisation of otherwise absent signal (RBM1 test 3) involved biotin label and a lower cycle number (10). Cycling could benefit from an increased primer concentration (267 tests 29, 30, 38, 39), although increasing unique target primer failed to achieve amplification (DAZ tests 1, 2).

TSA did produce an increase in D3Z1 signal but also heavy background (tests 30, 31). Anti-DIG antibody was therefore reduced to attempt to control production of extra signal (267 tests 13, 34, RBM1 cycling tests 2, 4, DAZ cycling test 3). Though 267 signal was somewhat enlarged TSA continued to produce extensive green 'haze' and failed to allow specific signal visualisation with unique target primers (though TSA could not be expected to work where cycling had caused damage to the preparation). This suggested that optimisation of TSA would require considerable modification of the recommended conditions.

It was observed that slight increase in slide age might affect outcome. Primer 267 test 26 on a 3 day-old slide used the same conditions as test 24 (1 day-old slide) and produced generally similar results. However test 4 (3 day slide, pre-fixed, 15pmoles primer) had fewer signals than test 7, a 1 day-old slide with less primer. Similarly, 10pmoles of primer on a 6 day-old slide produced less signal than 5pmoles on a 3 day slide (tests 22, 26). The use of 10 day-old slides apparently conferred no improvement with or without ligase treatment (D3Z1 tests 24 to 27). The best cycling and non-cycling results (e.g. 267 tests 31, 27) were obtained on 3 day-old slides.

There was no evidence in this study (e.g. 267 test 36, RBM1 test 6) that ligase treatment (as in Gosden and Hanratty, 1993) could reduce nonspecific background signal. Its use did appear to improve cycling results, while not removing 'haze', with D3Z1 primers on a 3-day but not a 10-day slide (tests 27, 25).

Pretreatment in fix then 2xSSC appeared to improve penetrance, increasing signal, as in 267 tests 9 and 11 or 10 and 12. Signal number and strength with TSA was also enhanced with this treatment (tests 13-16).

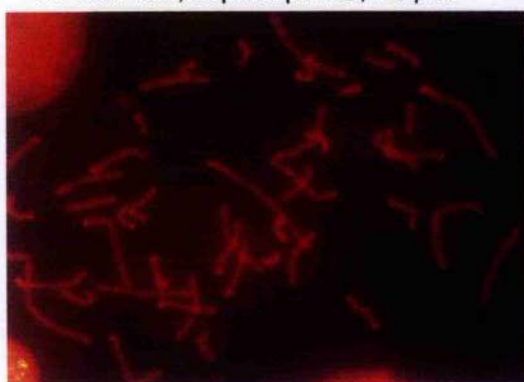
Proteinase K treatment at concentrations used in CGH caused damage on non-cycling PRINS with D3Z1 primers. Reducing proteinase K incubation time could, unexpectedly, produce a 'cleaner' preparation than that obtained with fix/2xSSC treatment (267 test 5) though this could have been due, rather, to the removal of fix/2xSSC.



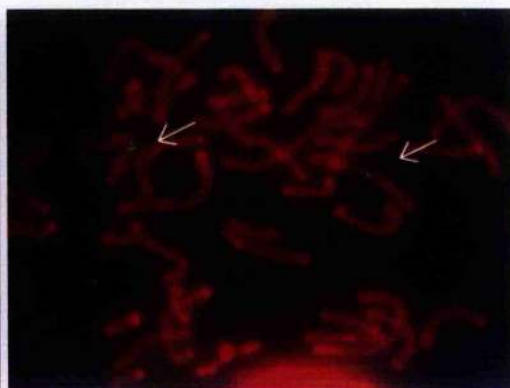
A. Test 4: No formamide denaturation, 68°C anneal, 50pmol primer, Taq I/S.



B. Test 7: As test 4, 15 cycles.



C. Test 24: As test 27, 10-day slide, 10 cycles, no ligase.



D. Test 27: 25pmol primer, 68°C anneal, Dynazyme, 3-day slide, 20 cycles, ligase.

Fig.3-25 D3Z1 PRINS images. Specific signals on chromosome 3 arrowed.

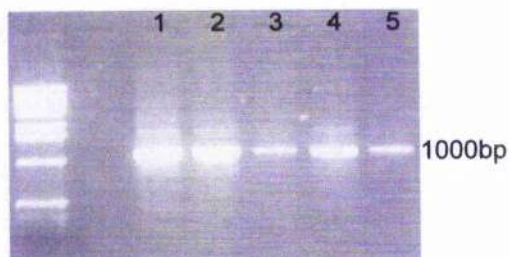


A. Without TSA.



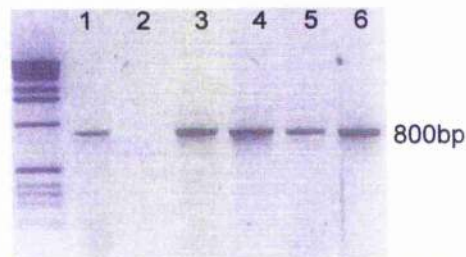
B. With TSA.

Fig.3-26 H1012 FISH images with and without TSA. Specific signals on chromosome 22 arrowed.



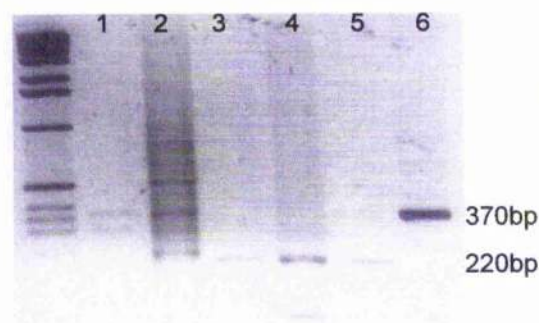
A: D3Z1 primers (Table 3-29).

1. Test 1: 50pmol primer, 1.5mM MgCl₂, Taq /S
2. Test 2: As 1 but 1mM MgCl₂
3. Test 3: As 1 but 25pmol primer
4. Test 4: As 2 but 25pmol primer
5. Test 5: As 3 but Dynazyme



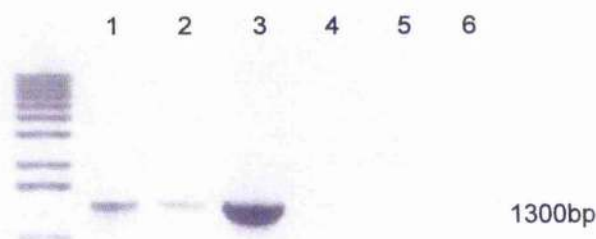
B: RBM1/YRRM primers (Table 3-36).

1. Test 3: 100uM dNTP, 1.5mM MgCl₂, Taq /S
2. Test 4: As test 3 but no enzyme
3. Test 5: 200uM dNTP, 4.5mM MgCl₂, Dynazyme
4. Test 6: As test 5 but 1.5mM MgCl₂
5. Test 7: As test 5 but 100uM dNTP
6. Test 8: As test 6 but 100uM dNTP



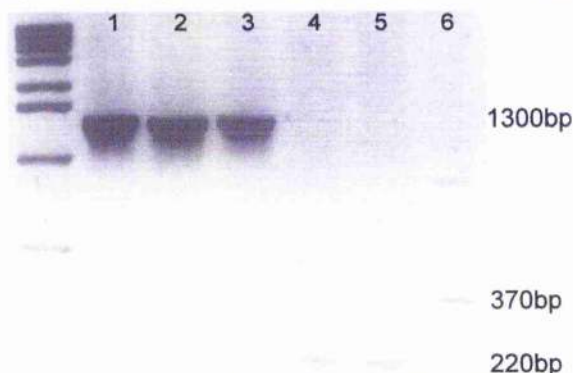
C: Factor IX (fIX) and DMD exon 6 primers. (Tables 3-31 and 3-33).

1. fIX test 6 : 50pmol primer, 1.5mM MgCl₂, Taq /S, 58°C anneal
2. fIX test 7: As fIX test 6 but 4.5mM MgCl₂
3. DMD exon 6 test 4: 50pmol primer, 1.5mM MgCl₂, Taq /S, 58°C anneal
4. DMD exon 6 test 5: As DMD exon 6 test 4 but 4.5mM MgCl₂
5. DMD exon 6 test 3: As DMD exon 6 test 4 but Dynazyme
6. fIX test 8: 20pmol primer, 1.5mM MgCl₂, Dynazyme, 63°C anneal



D: DAZ and DMD exon 6 primers (Tables 3-34 and 3-33).

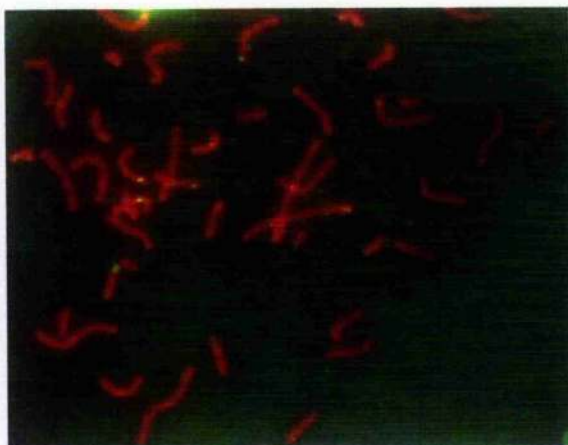
1. DAZ test 1: AmpliTaq (buffer contains MgCl₂)
2. DAZ test 3: 0.2U/ul Taq /S, 4.5mM MgCl₂
3. DAZ test 4: Dynazyme + Dynazyme buffer (contains MgCl₂)
4. DAZ test 5: Dynazyme, no detergent, 1.5mM MgCl₂
5. DAZ test 6: AmpliTaq, no detergent, 1.5mM MgCl₂
6. DMD exon 6 test 6: Dynazyme, no detergent, 1.5mM MgCl₂



E: DAZ, DMD exon 6 and Factor IX primers (Tables 3-34, 3-33 and 3-31).

1. DAZ test 7: 1.5mM MgCl₂, buffer+0.01% Triton X, Dynazyme
2. DAZ test 8: 1.5mM MgCl₂, buffer+0.02% Triton X, Dynazyme
3. DAZ test 4: Dynazyme + Dynazyme buffer (contains MgCl₂)
4. and 5. DMD exon 6 tests 7 and 8: 4.5mM MgCl₂, 0.2U/ul Taq /S, 50 or 100pmol primer respectively
6. Factor IX test 9: 20pmol primer, 1.5mM MgCl₂, Dynazyme, 63°C anneal

Fig.3-27 Images of gel electrophoresis following solution PCR.
Unmarked lane on the left of each gel contains a 1kb ladder.

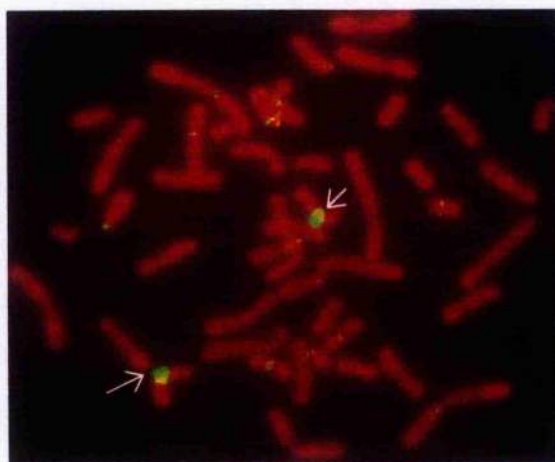


A. Test 5: 30 cycles.

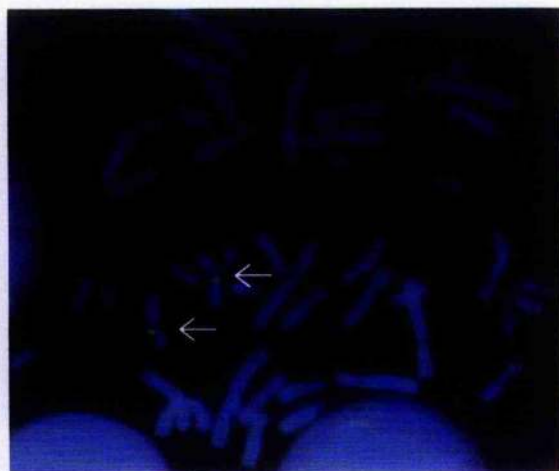


B. Test 6: 30 cycles, no enzyme.

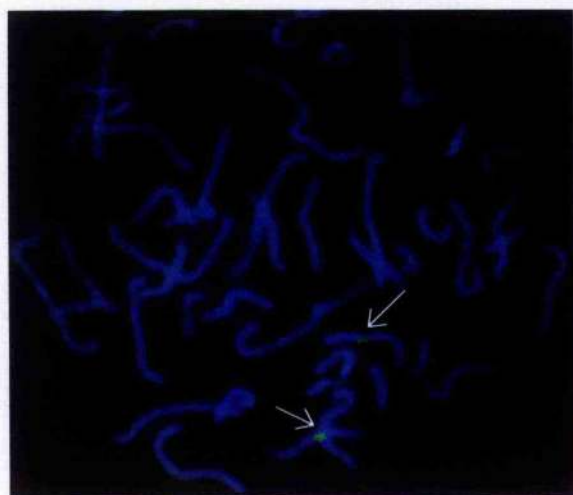
Fig.3-28 DMD PRINS images.



A. Test 3: 25pmol primer, 60°C anneal.



B. Test 27: 3pmol primer, 63°C anneal, reduced dNTPs.

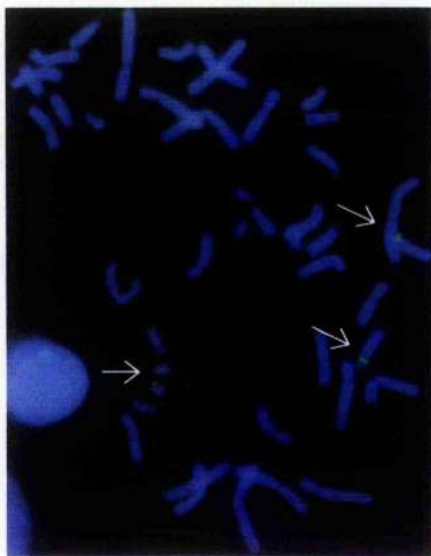


C. Test 31: 5 cycles 65°C anneal, 3pmol primer

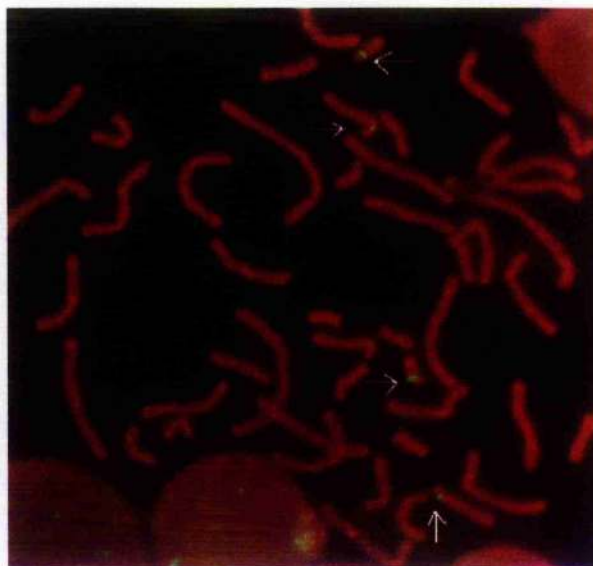


D. Test 34: As test 31 but no cycling, with TSA.

Fig.3-29 267 PRINS images. Signals on chromosome 9 arrowed.



13A



21A

Fig.3-30 13A and 21A PRINS images. Signals on chromosomes 13 and 21 arrowed.



Fig.3-25 Omnigene thermal cycler with flatbed module for slides (Hybaid).

Print Primer T	Buffer	label uM	dNTP uM	dTTP uM	MgCl ₂ mM	Enzyme	denat	anneal	extend	FITC layers	RESULT /COMMENTS	nuclei or metaphases	Signals
1	5ul /50ul	in mix	Bio-16 -dUTP label B-M mix 20uM (AB)	Print label mix (AB)	In mix	CPP (AB) 0.1U/ul	93°C 2'	60°C 10'	72°C 15'	1	Slide 1 day old. Pre-heated block to 93°C prior to slide addition. Signal weak, especially on nuclei. Background quite low.	Nuclei	54 34 24 2 0 1 2 3 21%
2	5ul /50ul	PE II	B-M DIG label mix 35uM	B-M DIG label mix 100uM	1mM	Taq /S 0.1U/ul	As 1	As 1	As 1	2	Signals present on more nuclei, but too weak to score. Low background. Slight background in metaphases, mainly on C-group chromosomes. Variation over slide.	Nuclei	44 22 30 1 30%
3	5ul /50ul	in mix	B-M DIG 20uM	Print label mix (AB)	In mix	CPP (AB) 0.1U/ul	As 1	As 1	As 1	2	Stronger signal, (possibly because buffer/enzyme used optimised for Prints). Slight background, mainly on C- group chromosomes. More speckle than 2.	Nuclei	19 36 50 4 50%
4	3ul /30ul	PE II	B-M DIG label mix 35uM	B-M DIG label mix 100uM	1mM	Taq /S 0.13U/ul	As 1	As 1	As 1	2	Strong specific signal, very faint background signal but heavy background speckling.	Nuclei	4 34 64 0 64%
												Metaphases	- - -

Table 3-27 PRINS with Chromosome 12 Print primers.

Denat: Denature
Print label mix (AB): Print label mix, Advanced Biotechnologies
Taq /S: AmpliTaq /S Polymerase 20U/ul, Roche Molecular Diagnostics
PE II: Buffer II x10, supplied in Perkin Elmer GeneAmp kit (100mM Tris-HCl, 500mM KCl, pH8.3)
B-M DIG label mix: Boehringer Mannheim Digoxigenin label mix (1mM dATP/dCTP/dGTP, 0.35mM dTTP, 0.35mM dUTP-DIG)
CPP (AB): Chromosome Print Polymerase 5U/ul, Advanced Biotechnologies
B-M DIG: Boehringer Mannheim Digoxigenin-11'-dUTP, 1mM
Bio-16-dUTP B-M: Biofin-16-dUTP, Boehringer Mannheim, 1mM
MgCl₂: Perkin Elmer, 25mM

Primer	Buffer	DIG	dNTP	dTTP	MgCl ₂	Enzyme	Equip	70%F	denat	anneal	extend	Cycle	FITC	RESULT/COMMENTS
pmol/ 30ul		-11 -dUTP	uM	uM	mM			denat- uration		°C		no.	layers	
1	50pmol 300ng	B-M PRINS kit	B-M PRINS kit	45uM	in buffer	B-M 0.084 U/ul	hot plates	NO preheat to 94°C 1' before add mix	91-94° 3'	60° 30'		1	2	Good signal/high background. Temperatures given are those on slide surface.
2	50pmol	PE II	B-M label mix	65uM in label mix + 60uM	1mM	Taq /S 0.2U/ul	Hybaid flatbed	NO preheat to 94°C 1' before add mix	95°C 3'	60°C 30'		1	2	Good signal: 5/5 cells with signal both 3s. 1/5 with signal also on chromosome 1cen. Less speckle background.
3	50pmol	B-M	B-M label mix	65uM in label mix + 60uM	0.33 mM	B-M 0.084 U/ul	Hybaid flatbed	NO	95°C 3'	60°C 30'		1	2	4/5 cells with signal on only both 3s. Speckle background.
4	50pmol	PE II	B-M 200uM each	100uM	1mM	Taq /S 0.2U/ul	Hybaid flatbed	NO	95°C 3'	60°C 30'		1	2	5/5 cells with signal on only both 3s. Only slight speckle background. Omitting slide preheat has produced no adverse effect.
5	50pmol	PE II	B-M 100uM	100uM	1mM	Taq /S 0.2U/ul	Hybaid flatbed	NO	95°C 3'	60°C 30'		1	2	Signal paler on metaphases than in test 4, but still reliable. No speckle background. But most nuclei with only very small or absent signal. 88 nuclei: 0 signal/ 7 nuclei: 1 signal/ 5 nuclei: 2 signals
6	50pmol	PE II	B-M 100uM	100uM	1mM	Taq /S 0.2U/ul	Hybaid flatbed	NO	95°C 3'	60°C 10'	72°C 15'	1	2	Increased extension temperature to 72°C. No signal on nuclei or metaphases.
7	50pmol	PE II	B-M 100uM	100uM	1mM	Taq /S 0.2U/ul	Hybaid flatbed	NO	95°C 3' then 95°C 30s	60°C 3'	65°C 10'		2	Nice signal, mostly just on chromosomes 3. Some background FITC speckle - like 'haze'. Control with no enzyme shows no signal, no 'haze'.
8	50pmol	PE II	B-M 100uM	100uM	1mM	Taq /S 0.2U/ul	Hybaid flatbed	YES		60°C 3'	65°C 10'		2	Signals OK. Some background signals. Some background FITC speckle - like 'haze'. Control with no enzyme shows no signal, no 'haze'.

Table 3-28 PRINS with D3Z1 alpha satellite primers (continued overleaf).

9	50pmol	PE I	B-M label mix 14uM	B-M label mix 40uM	26uM in label mix	In buffer	Ampli Taq 0.036 U/ul	Hybaid flatbed	YES 72°C 10'	60°C 10'	65°C 30'	1	2	Control for Cinti et al (1993) method test. 10 day-old slides. Centromere signal and background speckle though half recommended dNTPs/label used (Cinti did not use recommended 1:10 dNTP dilution) but, no added dTTP.
10	50pmol	PE II	B-M label mix 14uM	B-M label mix 40uM	26uM in label mix	NONE	Ampli Taq 0.036 U/ul	Hybaid flatbed	YES 72°C 10'	60°C 10'	65°C 30'	1	2	No signal. As Cinti et al (1993) - used no MgCl ₂ .
11	50pmol	PE II	B-M label mix 35uM	B-M label mix 100uM	65uM in label mix + 60uM	3mM	Taq /S 0.2U/ul	Hybaid flatbed	NO	95°C 3'	60°C 30'	1	2	Much more centromeric background signal than test 5. Background speckle.
12	50pmol	PE II	B-M 100uM	100uM each	60uM	1.5mM	Taq /S 0.2U/ul	Hybaid flatbed	NO	95°C 3'	60°C 30'	1	2	Much more centromeric background signal than test 5.
13	50pmol	PE II	B-M 100uM	100uM each	60uM	1.5mM	Taq /S 0.2U/ul	Hybaid flatbed	NO	95°C 3'	62°C 30'	1	2	Much more centromeric background signal than test 5.
14	50pmol	Gibco + 0.01% TritonX	B-M 100uM	100uM each	60uM	1.5mM	Dyna- zyme 0.04U/ul	Hybaid flatbed	NO	95°C 3'	60°C 30'	1	2	Even more background signal than test 12.
15	50pmol	Dyna- zyme	B-M 100uM	100uM each	60uM	1.5mM	Dyna- zyme 0.04U/ul	Hybaid flatbed	NO	95°C 3'	60°C 30'	1	2	Even more background signal than test 12 and much speckle background.
16	25pmol 150ng	Gibco + 0.01% TritonX	B-M 67uM	100uM each	100uM	1.5mM	Dyna- zyme 0.04U/ul	Hybaid flatbed	NO	95°C 3'	64°C 25'	1	2	Still too much background signal and speckle.
17	25pmol	Gibco + 0.01% TritonX	B-M 67uM	100uM each	100uM	1.5mM	Dyna- zyme 0.04U/ul	Hybaid flatbed	NO	95°C 3'	64°C 25'	1	1	Possibly less centromeric background than test 16.
18	25pmol	PE II	B-M 100uM	100uM each	100uM	1.5mM	Taq /S 0.2U/ul	Hybaid flatbed	NO	95°C 3'	64°C 25'	1	2	Still too much background signal and speckle.

19	25pmol	PE II	B-M 100uM	100uM each	100uM	1.5mM	Taq /S 0.2U/ul	Hybaid flatbed	NO	95°C 3'	64°C 25'	1	1	Possibly less centromeric background than test 18.
20	25pmol	PE II	B-M 100uM	100uM each	100uM	1.5mM	Taq /S 0.2U/ul	Hybaid flatbed	NO	92°C 3'	66°C 25'	1	1	Control for TSA test 30. Less centromeric background than test 19.
21	25pmol	Gibco + 0.01% TritonX	B-M 33uM	100uM each	100uM	1.5mM	Dyna- zyme 0.04U/ul	Hybaid flatbed	NO	92°C 3'	66°C 25'	1	1	Control for TSA test 31. Signal on 3, still some speckle.
22	25pmol	Gibco + 0.01% TritonX	B-M 33uM	100uM each	100uM	1.5mM	Dyna- zyme 0.04U/ul	Hybaid flatbed	NO	92°C 3'	68°C 25'	1	1	Signal on 3, but still speckle and occasional centromeric background.
23	25pmol	Gibco + 0.01% TritonX	B-M 17uM	100uM each	100uM	1.5mM	Dyna- zyme 0.04U/ul	Hybaid flatbed	NO	92°C 3'	68°C 25'	1	1	No signal.
24	25pmol	Gibco + 0.01% TritonX	B-M 33uM	100uM each	100uM	1.5mM	Dyna- zyme 0.04U/ul	Hybaid flatbed	NO	92°C 3' then 92°C 1'	68°C 1'	10	1	10 day-old slide with no ligase. No signal.
25	25pmol	Gibco + 0.01% TritonX	B-M 33uM	100uM each	100uM	1.5mM	Dyna- zyme 0.04U/ul	Hybaid flatbed	NO	As 24	As 24	10	1	10 day-old slide with ligase. No signal.
26	25pmol	Gibco + 0.01% TritonX	B-M 33uM	100uM each	100uM	1.5mM	Dyna- zyme 0.04U/ul	Hybaid flatbed	NO	As 24	As 24	10 or 20	1	3 day-old slide with no ligase. No signal.
27	25pmol	Gibco + 0.01% TritonX	B-M 33uM	100uM each	100uM	1.5mM	Dyna- zyme 0.04U/ul	Hybaid flatbed	NO	As 24	As 24	10 or 20	1	3 day-old slide with ligase. 'Haze' around metaphases. Specific signal on 80% chromosomes 3, no background signal. Signal retained after 20 cycles.

28	15pmol 90ng	Gibco + 0.01% TritonX	B-M 33uM	100uM each	1.5mM	Dyna- zyme 0.04U/uI	Hybaid flatbed	NO	92°C 3'	68°C 25'	1	1	3 day-old slide with ligase. Pale but reliable signals on all chromosomes 3.
29	15pmol	Gibco + 0.01% TritonX	B-M 33uM	100uM each	1.5mM	Dyna- zyme 0.04U/uI	Hybaid flatbed	NO	92°C 3'	68°C 25'	1	1	3 day-old slide with ligase plus proteinase K pretreatment (7.5 minutes, 37°C). Damage to nuclei, metaphases destroyed.
30	25pmol	PE II	B-M 100uM	100uM each	1.5mM	Taq /S 0.2U/uI	Hybaid flatbed	NO	92°C 3'	68°C 25'	1	1 + TSA	Bright signals on all chromosomes. Test 20 is the control (no TSA).
31	25pmol	Gibco + 0.01% TritonX	B-M 33uM	100uM each	1.5mM	Dyna- zyme 0.04U/uI	Hybaid flatbed	NO	92°C 3'	68°C 25'	1	1 + TSA	Bright signals on all chromosomes. Test 21 is the control (no TSA).

Table 3-28 PRINS with D3Z1 alpha satellite primers.

Denat: Denature

Gibco Buffer: Gibco BRL BufferX10; Life Technologies (200mM Tris-HCl, 500mM KCl, pH8.4, W-1 detergent supplied separately for use if required)

Dynazyme Buffer: Dynazyme reaction buffer x10, Finnzymes (100mM Tris-HCl pH8.3, 500mM KCl, 1% Triton-X 100)

Dynazyme: Dynazyme I Polymerase 2U/uI, Finnzymes

Taq /S: AmpliTaq /S Polymerase, 20U/uI, Roche Molecular Diagnostics

AmpliTaq: AmpliTaq Polymerase, 5U/uI, Roche Molecular Diagnostics

B-M label mix: Boehringer Mannheim Digoxigenin label mix (1mM dATP/dCTP/dGTP, 0.65mM dTTP, 0.35mM dUTP-DIG)

PE II: Buffer II x10, supplied in Perkin Elmer GeneAmp kit, Roche Molecular Systems Inc (100mM Tris-HCl, 500mM KCl, pH8.3)

MgCl₂: Perkin Elmer, 25mM

PE I: Buffer I x10, Roche Molecular Systems Inc (100mM Tris-HCl, 500mM KCl, 15mM MgCl₂, pH8.3)

B-M PRINS kit reaction buffer: Boehringer Mannheim PRINS kit reaction buffer x10 (contains MgCl₂, other constituent details not supplied)

B-M PRINS kit DIG-11-dUTP and dNTPs: Boehringer Mannheim PRINS kit labelling mix x10 (500uM dATP/dCTP/dGTP and 50uM DIG-11-dUTP in 50% glycerol)

B-M Enzyme: Boehringer Mannheim Taq DNA Polymerase, 1U/uI

B-M Buffer: Boehringer Mannheim reaction buffer x10 (without MgCl₂, other constituent details not supplied)

B-M DIG-11-dUTP: Boehringer Mannheim Digoxigenin-11-dUTP, 1mM

Ligase: T4 DNA Ligase 1U/uI, Gibco BRL. Prepared as 0.5U/uI in ligation buffer.

Proteinase K: Proteinase K, Sigma, 0.2ug/ml in 20mM Tris, 2mM CaCl₂, pH 7.5

TSA: Tyramide Signal Amplification, NEN, DuPont

1' represents 1 minute

70% F: 70% formamide

T E S T	Primers pmol	Buffer	dNTP uM	MgCl ₂ mM	Enzyme	denat	anneal	extend	Cycle Number	RESULT/COMMENTS
1	50pmol	PE II	100uM	1.5mM	Taq IS 0.2U/ul	95°C 3' then 95°C 1'	60°C 3'			Very strong band at >1kb. ? Slightly nonspecific.
2	50pmol	PE II	100uM	1.0mM	Taq IS 0.2U/ul	As 1	As 1		20	Strong band at >1kb (very slightly less than test 1). ? Slightly nonspecific.
3	25pmol	PE II	100uM	1.5mM	Taq IS 0.2U/ul	As 1	As 1		20	Band far weaker than test 1, but satisfactory. Totally specific.
4	25pmol	PE II	100uM	1.0mM	Taq IS 0.2U/ul	As 1	As 1		20	Stronger band than test 3, weaker than 2. Very slightly nonspecific.
5	25pmol	Gibco + 0.01% TritonX100	100uM	1.5mM	Dynazyme 0.04U/ul	As 1	As 1		20	As test 3.
6	25pmol new batch	Gibco + 0.01% TritonX100	100uM	1.5mM	Dynazyme 0.04U/ul	As 1	As 1		20	As test 5 - reproducible.

Table 3-29 Solution PCR with D3Z1 primers.
See D3Z1 PRINS Table 3-28 legend for explanatory notes.

T E S T	Primer pmol/ 50ul	Buffer	DIG -11 -dUTP	dNTP uM	dTTP uM	MgCl ₂ mM	Enzyme	Slide age (days)	70°F denat- uration	denat anneal extend	Cycle no.	FTC layers	RESULT/COMMENTS
	200 pmol	PE II	B-M label mix 14uM	B-M label mix 40uM	B-M label mix 26uM	NONE	Ampli Taq 0.04U/ul	10	YES	58°C 10' 72°C 30'	1	1	Cirti et al (1993) method. Avoided pre-heating slide and reaction mix, stop buffer omitted. 1:25 rather than 1:10 dilution of BM label mix.
1	200 pmol evapor -ated	PE II	B-M label mix 14uM	B-M label mix 40uM	B-M label mix 26uM	NONE	Taq /S 0.2U/ul	1	NO	95°C 3' 58°C 1' 72°C 3'	1	2	Pre-warmed slide and mix (minus enzyme) to 70°C. No signal, no background speckle. NB no MgCl ₂ .
2	200 pmol evapor -ated	PE II	B-M label mix 14uM	B-M label mix 40uM	B-M label mix 26uM	NONE	Taq /S 0.2U/ul	1	NO	95°C 3' 58°C 1' 72°C 3'	30	2	Pre-warmed slide and mix as in test 1. Nonspecific speckling plus ? signal on centromeres. Damage during cycling can cause mispriming but absence of MgCl ₂ would prevent enzyme activity.
3	200 pmol precipit -ated	PE II	B-M label mix 14uM	B-M label mix 40uM	B-M label mix 26uM	NONE	Ampli Taq 0.04U/ul	10	YES	58°C 10' 72°C 30'	1	2	No signal, no background speckle. D3Z1 controls, plus and minus MgCl ₂ , included - only worked <u>with</u> MgCl ₂ .
4	200 pmol precipit -ated	PE I	B-M label mix 14uM	B-M label mix 40uM	B-M label mix 26uM	in buffer	Ampli Taq 0.04U/ul	10	YES	58°C 10' 72°C 30'	1	2	Much background speckle. No definite signal. Possible signal on one chromosome X at distal Xq.

Table 3-30 PRINS with Factor IX primers.
See D3Z1 PRINS Table 3-28 legend for explanatory notes.

T E S T	Primers pmol	Buffer	dNTP uM	MgCl ₂ mM	Enzyme	denat °C	anneal °C	extend °C	Cycle Number	RESULT/COMMENTS
1	50pmol evapor- ated	Dynazyme	Pharmacia 200uM	in buffer 1.5mM	Dynazyme 0.04U/ul	95°C 1'	58°C 1'	72°C 2'	30	First cycle, denaturation 3'. Last cycle extension 10'. 72°C. Many nonspecific bands. DMD exon 6 control primers worked, with specific band at >200bp.
2	50pmol precipit- ated	Dynazyme	Pharmacia 200uM	in buffer 1.5mM	Dynazyme 0.04U/ul	95°C 1'	58°C 1'	72°C 2'	30	As Test 1.
3	50pmol precipit- ated	Dynazyme	PE 200uM	in buffer 1.5mM	Dynazyme 0.04U/ul	95°C 1'	58°C 1'	72°C 2'	30	As Test 1.
4	30pmol precipit- ated	Dynazyme	PE 200uM	in buffer 1.5mM	Dynazyme 0.04U/ul	95°C 1'	58°C 1'	72°C 2'	30	As Test 1.
5	30pmol precipit- ated	Dynazyme	PE 200uM	in buffer 1.5mM	Dynazyme 0.04U/ul	95°C 1'	61°C 1'	72°C 2'	30	As Test 1.
6	50pmol precipit- ated	PE II	PE 200uM	1.5mM	Taq /S 0.1U/ul	95°C 1'	58°C 1'	72°C 2'	30	Two pale bands - one, specific, at ~370bp.
7	50pmol precipit- ated	PE II	PE 200uM	4.5mM	Taq /S 0.1U/ul	95°C 1'	58°C 1'	72°C 2'	30	Stronger (nonspecific) bands than test 6.
8	20pmol precipit- ated	Dynazyme	PE 200uM	in buffer 1.5mM	Dynazyme 0.04U/ul	95°C 1'	63°C 1'	72°C 2'	30	Strong, specific band at ~370bp.
9	20pmol precipit- ated	Dynazyme	PE 200uM	in buffer 1.5mM	Dynazyme 0.04U/ul	95°C 1'	63°C 1'	72°C 2'	30	Strong, specific band at ~370bp plus nonspecific band.

Table 3-31 Solution PCR with Factor IX primers.
See D3Z1 PRINS Table 3-28 legend for explanatory notes.

I E T	Primer pmol/ 50ul	Buffer	DIG -11 -dUTP	dNTP uM	dTTP uM	MgCl ₂ mM	Enzyme	Equip -ment	70%F denat- uration	°C denat anneal extend	Cycle no.	FITC layers	RESULT/COMMENTS
1	exon51 50pmol	B-M PRINS kit	B-M label mix 35uM	B-M label mix 100uM	65uM in label mix	in buffer	B-M 0.084 U/ul	PE	NO	94°C 3' 58°C 1' 68°C 5'	12	1	No signal, very clean. 1 day-old, solarised, slide used. Pre-warmed mix and slide (70°C) prior to combining. ? Too little primer.
2	exon51 50pmol	B-M PRINS kit	B-M label mix 35uM	B-M label mix 100uM	65uM in label mix	in buffer	B-M 0.084 U/ul	PE	NO	94°C 3' 58°C 1' 68°C 5'	30	1	No signal, PRINS area degraded. No metaphases and very few nuclei remaining. Likely cause is detergent concentration in enzyme mix, also slides may be too new. ? Too little primer.
3	exon6 100 pmol	PE II	100uM	200uM	NONE	4mM	Taq /S 0.2U/ul	Hybaid	YES	58°C 3' 58°C 1' 68°C 3'	30	2	FITC speckle background everywhere. occasional large nonspecific signals. Membrane around metaphases. Quite a lot of nonspecific signals, mostly at cen and tel regions.
4	exon6 100 pmol	PE II	100uM	200uM	NONE	4mM	OMIT for control	Hybaid	YES	As 3 58°C As 3 58°C As 3 68°C As 3	30	2	Clean, occasional small, nonspecific, signals.
5	exon6 100 pmol	PE II	100uM	200uM	NONE	4mM	Taq /S 0.2U/ul	Hybaid	NO	95°C 3' 58°C 1' 68°C 3'	30	2	FITC speckle background. No membrane around metaphases. Quite a lot of nonspecific signals, mostly at cen and tel regions.
6	exon6 100 pmol	PE II	100uM	200uM	NONE	4mM	OMIT for control	Hybaid	NO	As 5 58°C As 5 58°C As 5 68°C As 5	30	2	Clean. Occasional small nonspecific signals.

Table 3-32 PRINS with Dystrophin primers.
See D3Z1 PRINS Table 3-28 legend for explanatory notes.

T S T	Primer pmol	Buffer	dNTP uM	MgCl ₂ mM	Enzyme	denat	anneal	extend	Cycle number	RESULT/COMMENTS
1	50pmol	Dyna- zyme	Pharmacia 200uM	in buffer 1.5mM	Dyna- zyme 0.04U/ul	95°C 3' then 95°C 1'	58°C 1'	72°C 2'	1	Final extension 10'. Specific, band at 200-220bp.
2	50pmol	Dyna- zyme	PE 200uM	in buffer 1.5mM	Dyna- zyme 0.04U/ul	As 1	As 1	As 1	As 1	
3	50pmol	Dyna- zyme	PE 200uM	in buffer 1.5mM	Dyna- zyme 0.04U/ul	As 1	As 1	As 1	As 1	
4	50pmol	PE II	PE 200uM	1.5mM	Taq /S 0.1U/ul	As 1	As 1	As 1	As 1	
5	50pmol	PE II	PE 200uM	4.5mM	Taq /S 0.1U/ul	As 1	As 1	As 1	As 1	Slightly stronger band at 200-220bp than in test 1. An additional, weaker, more distal, band present.
6	50pmol	Gibco	PE 200uM	1.5mM	Dyna- zyme 0.04U/ul	As 1	As 1	As 1	As 1	No amplification - no detergent.
7	50pmol	PE II	PE 200uM	4.5mM	Taq /S 0.2U/ul	As 1	As 1	As 1	As 1	Increased Taq /S concentration. As 1 - would expect 'As 5' since 4.5mM MgCl ₂ .
8	100pmol	PE II	PE 200uM	4.5mM	Taq /S 0.2U/ul	As 1	As 1	As 1	As 1	Increased Taq /S and primer concentration. As 7

Table 3-33 Solution PCR with Dystrophin exon 6 primers.
See D3Z1 PRINS Table 3-28 legend for explanatory notes.

T E S T	Primers pmol	Buffer	dNTP uM	MgCl ₂ mM	Enzyme	denat °C	anneal °C	extend °C	Cycle Number	RESULT/COMMENTS
1	30pmol	PE I	200uM	in buffer	AmpliTaq 0.05U/ul	95°C 3'	58°C 1'	72°C 2'	1	Final extension at 72°C 10'. Very strong band ~1300bp. Specific. Repeated as control with tests 3-6, same result.
2	30pmol	PE II	200uM	4.5mM	Taq /S 0.1U/ul	As 1	As 1	As 1	As 1	Weak, specific band.
3	30pmol	PE II	200uM	4.5mM	Taq /S 0.2U/ul	As 1	As 1	As 1	As 1	Stronger band than test 2.
4	30pmol	Dynazyme	200uM	in buffer	Dynazyme 0.04U/ul	As 1	As 1	As 1	As 1	Strong, specific band. Repeated as control for tests 7 and 8.
5	30pmol	Gibco No detergent	200uM	1.5mM	Dynazyme 0.04U/ul	As 1	As 1	As 1	As 1	FAIL. No detergent (except 0.1% Triton-X 100 in the 1ul enzyme). Repeated as control for tests 7 and 8.
6	30pmol	Gibco No detergent	200uM	1.5mM	AmpliTaq 0.05U/ul	As 1	As 1	As 1	As 1	FAIL. No detergent (except 0.1% Triton-X 100 in the 1ul enzyme).
7	30pmol	Gibco + 0.01% Triton X-100	200uM	1.5mM	Dynazyme 0.04U/ul	As 1	As 1	As 1	As 1	Even stronger band than test 4.
8	30pmol	Gibco + 0.02% Triton X-100	200uM	1.5mM	Dynazyme 0.04U/ul	As 1	As 1	As 1	As 1	As test 7.

Table 3-34 Solution PCR with DAZ primers.
See D3Z1 PRINS Table 3-28 legend for explanatory notes.

T E S T	Primers pmol/ 50ul	Buffer	DIG -11 -dUTP	dNTP uM	dTTP uM	MgCl ₂ mM	Enzyme	Equip -ment	70%F denat- uration	°C denat	anneal	extend	Cycle no.	FITC layers	RESULT/COMMENTS
1	30 pmol	PE II	B-M label mix 35uM	B-M label mix 100uM	65uM in label mix	4.5mM	Taq IS 0.2U/ul	Hybaid flatbed	NO	95°C 3' then 95°C 1'	58°C 1'	72°C 2'	1	2	Block and wash with TSA system reagents but no TSA. Clouds of fluorescent speckle. Metaphases destroyed (absent) or very pale.
2	100 pmol	PE II	B-M label mix 35uM	B-M label mix 100uM	65uM in label mix	4.5mM	Taq IS 0.2U/ul	Hybaid flatbed	NO	As 1	As 1	As 1	31	2	As 1
3	30 pmol	PE II	B-M label mix 35uM	B-M label mix 100uM	65uM in label mix	4.5mM	Taq IS 0.2U/ul	Hybaid flatbed	NO	As 1	As 1	As 1	31	1 + TSA	No clouds of speckle around metaphases. Very bright, convincing but spurious, deposits on a few chromosomes.

Table 3-35 Cycling PRINS with DAZ Primers.

See D3Z1 PRINS Table 3-28 legend for explanatory notes.

T E S T	Primers pmol	Buffer	dNTP uM	MgCl ₂ uM	Enzyme	denat.	°C anneal	°C extend	Cycle Number	RESULT/COMMENTS
1	30pmol	PE I	200uM	in buffer	AmpliTaq 0.05U/ul	95°C 3' then 95°C 1'	58°C 1'	72°C 2'	1	Final extension at 72°C, 10'. Strong band ~800bp. Specific.
2	30pmol	PE II	200uM	4.5mM	Taq /S 0.1U/ul	As 1	As 1	As 1	As 1	Weaker than 1, but still strong, specific band.
3	30pmol	PE II	100uM	4.5mM	Taq /S 0.2U/ul	As 1	As 1	As 1	As 1	By far the weakest band, but amplification adequate. Very specific.
4	30pmol	PE II	100uM	4.5mM	NONE tube control	As 1	As 1	As 1	As 1	No amplification. Enzyme omitted for tube control.
5	30pmol	Gibco + 0.01% Triton X-100	200uM	4.5mM	Dynazyme 0.04U/ul	As 1	As 1	As 1	As 1	Very slightly weaker band than test 6. More intense than test 3.
6	30pmol	Gibco + 0.01% Triton X-100	200uM	1.5mM	Dynazyme 0.04U/ul	As 1	As 1	As 1	As 1	Very slightly stronger than test 5. More intense than test 3.
7	30pmol	Gibco + 0.01% Triton X-100	100uM	4.5mM	Dynazyme 0.04U/ul	As 1	As 1	As 1	As 1	Weaker band than tests 5 or 6. Similar to test 2. Very clean.
8	30pmol	Gibco + 0.01% Triton X-100	100uM	1.5mM	Dynazyme 0.04U/ul	As 1	As 1	As 1	As 1	Similar to test 6. Very clean.

Table 3-36 Solution PCR with RBM1/YRRM primers.
See D3Z1 PRINS Table 3-28 legend for explanatory notes.

T E S T	Primer pmol/ 30ul	Buffer	label uM	dNTP uM	dTTP uM	MgCl ₂ mM	Enzyme	Equip -ment	70%F denat- uration	denat	anneal	extend	Cycle no.	FITC layers	RESULT/COMMENTS
1	30pmol	PE II	B-M DIG label mix 35uM	B-M DIG label mix 100uM	65uM in label mix	4.5mM	Taq /S 0.2U/ul	Hybaid flatbed	NO	95°C 3' then 95°C 1'	58°C 1'	72°C 2'	1	2	Final extension at 72°C, 10'. Block and wash with TSA system reagents but no TSA. Clouds of fluorescent speckle. Metaphases destroyed (absent) or very pale. If present, sited in dark 'hole' in FITC speckle.
2	30pmol	PE II	B-M DIG label mix 35uM	B-M DIG label mix 100uM	65uM in label mix	4.5mM	Taq /S 0.2U/ul	Hybaid flatbed	NO	As 1	As 1	As 1	As 1	TSA	Final extension at 72°C, 10'. No clouds of speckle around metaphases. Very bright, convincing though spurious, deposits on a few chromosomes.
3	30pmol	PE II	B-M Bio-16 -dUTP 100uM	100uM	NONE	4.5mM	Taq /S 0.2U/ul	Hybaid flatbed	NO	As 1	As 1	As 1	11	2	Placed on block at 95°C. Final extension at 72°C, 10'. 1 day-old slides. Very few signals. Clouds of fluorescent speckle over metaphases. Three out of four metaphases showed some signal on Y.
4	30pmol	PE II	B-M Bio-16 -dUTP 100uM	100uM	NONE	4.5mM	Taq /S 0.2U/ul	Hybaid flatbed	NO	As 3	As 3	As 3	As 3	TSA	Placed on block at 95°C. Final extension at 72°C, 10'. 1 day-old slides. Cloudy, chromosomes becoming pale. No signals.
5	30pmol	Gibco + 0.01% Triton X-100	B-M DIG-11 -dUTP 100uM	200uM	NONE	1.5mM	Dyna zyme 0.04U/ul	Hybaid flatbed	NO	92°C 3' then 92°C 1'	57°C 1'	71°C 2'	1	2	No ligase pretreatment. 10 day-old slide. 71°C 10' final extension. As above, masses of fluorescent speckle over metaphases, not between metaphases. Metaphases very pale.
6	30pmol	Gibco + 0.01% Triton X-100	B-M DIG-11 -dUTP 100uM	200uM	NONE	1.5mM	Dyna zyme 0.04U/ul	Hybaid flatbed	NO	As 5	As 5	As 5	As 5	As 5	Ligase pretreatment. 10 day-old slide. 71°C 10' final extension. As test 5.

Table 3-37 Cycling PRINS with RBM1/YRRM Primers.

See legends on chromosome 12 Print and D3Z1 PRINS Tables 3-27 and 3-28 for explanatory notes.

T E S T	Primer	DIG -11 -dUTP	dNTP uM	dTTP uM	MgCl ₂ mM	Enzyme/ Buffer	SLIDE AGE (days)	LIGASE FIX 60/ 2XSSC 37°C 30'	denat anneal extend	°C	Cycle no.	FITC layers	RESULT/COMMENTS
* 50- 600ug/ 50ul		100uM	200uM	20uM	1.5mM	B-M 0.02U/ul PE II	<7	YES	70°F then 95°C 1'	60°C 3'	up to 30	1	1 cycle 1cen, 1 pair acrocentrics, 9cen (weak) 10 cycles 12 sites 30 cycles 18 sites
1	50pmol/ 30ul	100uM	200uM	20uM	1.5mM	Dynazyme 0.04U/ul Gibco + TritonX- 100	1	YES	95°C 3'	60°C 3'	1	1	13 signals - need to adjust conditions.
2	25pmol /30ul	100uM	100uM	20uM	1.5mM	As 1	1	NO	95°C 3'	60°C 3'	1	1	12-15 signals, including 1cen.
3	25pmol /30ul	50uM	100uM	20uM	1.5mM	As 1	1	NO	95°C 3'	60°C 3'	1	1	12-17 signals (many weak) including 1cen.
4	15pmol /30ul	50uM	100uM	20uM	1.5mM	As 1	3	NO	95°C 3'	63°C 3'	1	1	9-12 signals, including 1cen and 7-8 acrocentric.
5	5pmol /30ul	33uM	33uM	10uM	1.5mM	As 1	3	NO	95°C 3'	63°C 3'	1	1	13 signals, including faint 1cen, 2 strong acrocentric, 6 weaker acrocentric, weak 9cen. Lots of background speckle, not just on chromosomes.
6	5pmol /30ul	33uM	33uM	10uM	1.5mM	As 1	3	NO	95°C 3'	55°C 3'	1	1	11 signals, including stronger 1cen than in test 5. Possibly even more background speckle than test 5.

Table 3-38 PRINS with 435 primers.

FIX: 3:1 methanol:acetic acid fixative; PK: Proteinase K (Sigma) 0.2ug/ml in 20mM Tris, 2mM CaCl₂, pH 7.5

See D3Z1 PRINS Table 3-28 legend for other explanatory notes.

* published conditions (Gosden and Hanratty 1993)

T E S T	Primer	DIG -11 -dUTP	dNTP uM	dATP uM	MgCl ₂ mM	Enzyme/ Buffer	SLIDE AGE (days)	LIGASE PK or FIX 60/ 2XSSC 37°C 30'	denat	°C		Cycle no.	FITC layers	RESULT/COMMENTS
										anneal	extend			
*	50- 500ug/ 50ul	100uM	200uM	20uM	1.5mM	B-M 0.02U/ul PE II	<7	YES	70°F	60°C 3'	70°C 10'	up to 30	1	1 cycle 9cen 5 cycles 14 signals: 9cen, 16cen, all acrocentrics
1	50pmol/ 30ul	100uM	200uM	20uM	1.5mM	Dynazyme 0.04U/ul Gibco + TritonX-100	1	YES	95°C 3'	60°C 3'	70°C 10'	1	1	28 signals - need to adjust conditions.
2	25pmol /30ul	100uM	100uM	20uM	1.5mM	As 1	1	NO	95°C 3'	60°C 3'	70°C 10'	1	1	15-18 signals: 9cen, 5cen, 19cen all x 2, 1cen x 1, 16cen, 7cen weak, ~all acrocentrics
3	25pmol /30ul	50uM	100uM	20uM	1.5mM	As 1	1	NO	95°C 3'	60°C 3'	70°C 10'	1	1	20 signals, though many weak, strong signal at 9cen.
4	15pmol /30ul	50uM	100uM	20uM	1.5mM	As 1	3	NO	95°C 3'	63°C 3'	72°C 10'	1	1	9cen, 'speckle' signal on other chromosomes (including 6-9 acrocentric).
5	15pmol /30ul	50uM	100uM	20uM	1.5mM	As 1	3	NO	95°C 3'	63°C 3'	72°C 10'	1	1	Only 9cen signal strong - others absent or weak.
6	10pmol /30ul	50uM	100uM	20uM	1.5mM	As 1	1	YES	95°C 3'	63°C 3'	72°C 10'	1	1	9-15 signals (9cen strong, most weak).
7	10pmol /30ul	50uM	100uM	20uM	1.5mM	As 1	1	YES	95°C 3'	63°C 3'	72°C 10'	1	1	~21 signals, most strong.
8	15pmol/ 30ul	50uM	100uM	30uM	1.5mM	As 1	1	YES	95°C 3'	63°C 3'	72°C 10'	1	1	11-13 signals (including 9cen, 5 acrocentric).

Table 3-39 PRINS with 267 primers (continued overleaf).

* published conditions (Gosden and Hanratty 1993)

9	10pmol/ 30ul	50uM	100uM	20uM	1.5mM	As 1	1	YES	FIX 60/ 2XSSC 37°C 30'	95°C 3' then 95°C 1'	63°C 3'	72°C 10'	5	1	11-13 signals (including 6-7 acrocentric) only 9cen strong.
10	15pmol/ 30ul	50uM	100uM	30uM	1.5mM	As 1	1	YES	FIX 60/ 2XSSC 37°C 30'	As 9	As 9	As 9	5	1	11-12 signals (including 9cen, 5-8 acrocentric) Weaker than non-cycled (? diffusion, ? heat damage).
11	10pmol /30ul	50uM	100uM	20uM	1.5mM	As 1	1	YES	NO	As 9	As 9	As 9	5	1	No signal (whereas fixed slide test 9 had signals).
12	15pmol/ 30ul	50uM	100uM	30uM	1.5mM	As 1	1	YES	NO	As 9	As 9	As 9	5	1	No signal (whereas fixed slide test 10 had signals).
13	10pmol /30ul	50uM	100uM	20uM	1.5mM	As 1	1	YES	NO	95°C 3'	63°C 3'	72°C 10'	1	TSA 1:100 anti- DIG	In metaphases, 8cen signals no stronger than in test 6. Signal distribution erratic: 9cen in one cell, acrocentric signal in one cell, 5cen in one cell. Signal bright on nuclei. As other TSA slides, green haze present.
14	10pmol /30ul	50uM	100uM	20uM	1.5mM	As 1	1	YES	FIX 60/ 2XSSC 37°C 30'	95°C 3'	63°C 3'	72°C 10'	1	TSA As13	15 signals (including strong 9cen) As other TSA slides, green haze present.
15	15pmol/ 30ul	50uM	100uM	30uM	1.5mM	As 1	1	YES	NO	95°C 3'	63°C 3'	72°C 10'	1	TSA As13	~ Same as 10pmol/no fix (test 13), but one cell with 8 strong signals, including 9cen and 3 acrocentric.
16	15pmol/ 30ul	50uM	100uM	30uM	1.5mM	As 1	1	YES	FIX 60/ 2XSSC 37°C 30'	95°C 3'	63°C 3'	72°C 10'	1	TSA As13	Stronger signal than 15 - one cell had 7 acrocentric signals.
17	10pmol /30ul	50uM	100uM	30uM	1.5mM	As 1	1	NO	FIX 60/ 2XSSC 37°C 30'	95°C 3'	66°C 3'	72°C 10'	1	1	Too many signals (strong 9cen). much background speckle.
18	10pmol /30ul	50uM	100uM	50uM	1.5mM	As 1	1	NO	FIX 60/ 2XSSC 37°C 30'	95°C 3'	66°C 3'	72°C 10'	1	1	Too many signals (weaker 9cen than test 17), background speckling worse than test 17.
19	5pmol /30ul	50uM	100uM	30uM	1.5mM	As 1	1	NO	FIX 60/ 2XSSC 37°C 30'	95°C 3'	66°C 3'	72°C 10'	1	1	10 signals (weaker 9cen than test 17, 1cen, 16cen, 5cen and ~4 acrocentric). Still considerable background speckling.

20	10pmol /30ul	50uM	100uM	100uM	1.5mM	As 1	1	NO	FIX 60/ 2XSSC 37°C 30'	95°C 3'	63°C 3'	72°C 10'	1	1	As test 19.
21	5pmol /30ul	33uM	50uM	10uM	1.5mM	As 1	6	NO	2XSSC 37°C 30'	95°C 3'	63°C 3'	72°C 10'	1	1	2-5 signals including 9cen (quite weak, often just visible with dual filter) ~2 acrocentric.
22	10pmol /30ul	33uM	33uM	10uM	1.5mM	As 1	5	NO	2XSSC 37°C 30'	95°C 3'	63°C 3'	72°C 10'	1	1	1-5 signals, including ~2 acrocentric.
23	5pmol /30ul	33uM	50uM	20uM	1.5mM	As 1	8	NO	2XSSC 37°C 30'	95°C 3'	63°C 3'	72°C 10'	1	1	2 weak signals (including 1 acrocentric). Expect less signal with more dTTP but reduced too much. NB acrocentric signal still persisting.
24	5pmol /30ul	33uM	33uM	10uM	1.5mM	As 1	1	NO	2XSSC 37°C 30'	95°C 3'	63°C 3'	72°C 10'	1	1	2-5 signals (and ~6 acrocentrics, 5cen, 19cen have weak specks). Like test 22 but with less primer and newer slide.
25	5pmol /30ul	33uM	50uM	20uM	1.5mM	As 1	1	NO	2XSSC 37°C 30'	95°C 3'	63°C 3'	72°C 10'	1	1	2-12 signals (including 5-7 acrocentric) plus much more speckle on and background around chromosomes than 24, though tests on same slide. Like 23 but newer slide.
26	5pmol /30ul	33uM	33uM	10uM	1.5mM	As 1	3	NO	2XSSC 37°C 30'	95°C 3'	63°C 3'	72°C 10'	1	1	2-14 signals, though most weak in cell with 14. Other cells with strong signals only on 9cen and ~2 acrocentric. More speckles than on one-day slide (test 24)
27	3pmol /30ul	33uM	33uM	10uM	1.5mM	As 1	3	NO	2XSSC 37°C 30'	95°C 3'	63°C 3'	72°C 10'	1	1	Only 9cen signals quite strong. 1-3 additional weak speckle signals. Same slide as test 30. Probably the best non-cycling result.
28	3pmol /30ul	33uM	33uM	10uM	1.5mM	As 1	3	NO	2XSSC 37°C 30'	95°C 3'	63°C 3'	72°C 10'	1	1	Signal on 9cen weaker than with 63°C anneal (test 27). Also on other sites but difficult to differentiate excessive background speckles over chromosomes from true signal.
29	5pmol /30ul	33uM	33uM	10uM	1.5mM	As 1	3	NO	2XSSC 37°C 30'	95°C 3'	63°C 3'	72°C 3'	5	1	0-3 signals, including 8cen signal (19cen also in one cell). Signal quite faint. Some 'haze' behind metaphases.
30	3pmol /30ul	33uM	33uM	10uM	1.5mM	As 1	3	NO	2XSSC 37°C 30'	95°C 1'	63°C 1'	72°C 3'	5	1	As 29, signal paler.

31	5pmol /30ul	33uM	33uM	10uM	1.5mM	As 1	3	NO	2XSSC 37°C 30'	95°C 3' then 95°C 1'	65°C 3'	72°C 3'	5	1	2-3 signals (9cen quite strong) plus speckle on ~2 acrocentrics in one cell. Probably the best cycling result.
32	3pmol /30ul	33uM	33uM	10uM	1.5mM	As 1	3	NO	2XSSC 37°C 30'	As 31	As 31	As 31	5	1	Only 1-2 faint signals (no signal in one cell).
33	5pmol /30ul	33uM	33uM	10uM	1.5mM	As 1	1	NO	NO	95°C 3'	65°C 3'	72°C 10'	1	1	No stop buffer - control for TSA test 34 in which it was also omitted. ~ 8 signals + speckles.
34	5pmol /30ul	33uM	33uM	10uM	1.5mM	As 1	1	NO	NO	95°C 3'	65°C 3'	72°C 10'	1	TSA As13	Signals on 9cen. A few other signals present (e.g. 1cen), some difficult to place because of green 'haze'. Signals possibly brighter and more diffuse with TSA. No cycling or stop buffer yet still have green 'haze'.
35	5pmol /30ul	33uM	33uM	10uM	1.5mM	As 1	1	NO	2XSSC 37°C 30'	95°C 3'	65°C 3'	72°C 10'	1	1	2 signals (9cen) plus much speckle background on chromosomes. Control for + Ligase test 36.
36	5pmol /30ul	33uM	33uM	10uM	1.5mM	As 1	1	YES	2XSSC 37°C 30'	95°C 3'	65°C 3'	72°C 10'	1	1	2-4 signals (including 9cen) plus much speckle background.
37	5pmol /30ul	33uM	33uM	10uM	1.5mM	As 1	1	YES	2XSSC 37°C 30'	95°C 3' then 95°C 1'	65°C 3'	72°C 3'	5	1	1 signal (9cen).
38	5pmol /30ul	33uM	33uM	10uM	1.5mM	As 1	1	NO	2XSSC 37°C 30'	As 37	As 37	As 37	10	1	Very pale, ~no signal.
39	20pmol /30ul	33uM	33uM	10uM	1.5mM	As 1	1	NO	2XSSC 37°C 30'	As 37	As 37	As 37	10	1	6 signals (including 9cen, ~3 acrocentric), some pale. Same slide as test 38.

Table 3-39 PRINS with 267 primers.

FIX: 3-1 methanol:acetic acid fixative; PK: Proteinase K (Sigma) 0.2ug/ml in 20mM Tris, 2mM CaCl₂, pH 7.5

anti DIG: horseradish peroxidase (HRP)-labelled anti-digoxigenin antibody

See D3Z1 PRINS Table 3-28 legend for other explanatory notes.

* published conditions (Gosden and Hanratty 1993)

4. DISCUSSION

4.1 Human DNA Sequence Mapping and Chromosomal Breakpoint Definition

The clone localisation, map confirmation and/or refinement performed during the course of this study was of substantial value to the collaborating scientists. By identifying restricted chromosomal loci it allowed consultation of, and comparison with, information in genetic databases. It also provided evidence that sequences were, or were not (as in the case of PAC227) likely to be the sequences they were believed to be. Identity confirmation allowed sequences to be used in further studies.

MRCK β , *SCD2* and *hTERT* Localisation

MRCK β or CDC42BPB is now known as CDC42 binding protein kinase beta (DMPK-like), Genbank Gene ID:9578. The map location first determined in this study, 14q32.31~q32.32, has been confirmed, and it is now known to lie 101.38-101.5Mb from the chromosome 4 short arm terminus (Ensembl).

Mapping the putative *SCD2* sequences to 4q21, rather than chromosome 10 (the *SCD* locus) or 17 (the locus of a transcriptionally inactive *SCD* pseudogene, Zhang et al 1999) gave some support to our collaborator's proposal that this sequence could represent a second *SCD* gene. However the existence of a second human *SCD* gene has not yet been confirmed, although a third mouse *Scd3* gene has now been identified (Zheng et al 2001). All three mouse *Scd* genes localise to mouse chromosome 19 which has reported synteny with human chromosome 10, but not with 4q21 (Gregory et al 2002).

hTERT co-localised with 5p subtelomeric probe sequence in this study, but it could actually lie up to ~1Mb distant because of limited resolution on the metaphase chromosomes probed. Also sequence ordering can be distorted at telomeric regions (Trask et al 1991). *hTERT* is now known to map 1.3Mb from the 5p telomere (Ensembl), however precise localisation information is not available for 5ptel sequence 114j18. It may be that mapping of the reverse transcriptase component of telomerase to a telomeric region could be relevant to the regulation of its expression by chromatin structure and telomere position effects (Bryce et al 2000).

Deletions at 5p15.3 are associated with cri du chat syndrome which is characterised by growth failure, microcephaly, facial abnormalities and severe retardation. It was hoped that the *hTERT* probe might assist definition of the genes clustering in this region and refining of deletion boundaries. Concomitant deletion at *hTERT* was demonstrated in all

ten patients examined in a recent study, and the authors propose that *hTERT* haploinsufficiency may contribute to the cri du chat phenotype (Zhang et al 2003). The widespread interest in the crucial role of *hTERT* in cellular immortalisation and tumorigenesis has resulted in the *hTERT* sequence mapped in this study being marketed commercially by Qbiogene.

Mapping and Ordering of Chromosome 21-Specific Probes for Breakpoint Definition in a Case of Partial Trisomy 21

Breakpoint mapping with 21q-specific YACs showed that the 21q region monosomic in individual DP was proximal to sequence RP11 268F23, which lies at 16.03Mb from 21pter (the 21 centromere extends to 13.2Mb according to Ensembl). Concurrent quantitative PCR studies by Dr Sandy Cooke of the molecular genetics division of this Institute determined that sequence D21S172 is included in the monosomic region. The breakpoint on at least one of the two chromosome 21 components of the derivative chromosome 12, initially defined as der(12)(12qter→12p13.3::21q22.3→21q11.2::21q11.2→21qter), therefore lies between these two loci, at 15.86-16.03Mb in 21q21.1. This work has provided a focussed region for further biochemical and molecular study. According to Ensembl, eighteen genes are sited in the region between sequence 268F23 and the centromeric heterochromatin.

One of the chromosome 21 components could have a more distal breakpoint (though proximal to 925h10/D21S1257) as it was not possible, by metaphase FISH, to discern if 268F23 was present in one or two copies on the der(12) chromosome, an important distinction in this study. Nuclear scoring for 268F23 sequence copy number determination might resolve this, but would require optimisation of the probe for interphase FISH. Instead, further quantitative PCR would possibly be a more straightforward approach, and would conserve the remaining cytogenetic preparation of patient cells.

Localisation and ordering data on the MPI YAC sequences was incidentally obtained prior to and during breakpoint determination studies. This information was relayed to the MPI database, resulting in their re-positioning of 937e12 distal to 856h02 and 858e10.

Accurate map locations for markers associated with the MPI sequences are now available and are presented in Table 4-1 with the mapping results from this study.

The co-localisation of 949b09 (at 15.06Mb) and 858e10 (15.8Mb) in all cells examined supports the finding that the resolution for ordering sequences by such two colour mapping is >1Mb (Trask et al 1991). Trask also observed that, even with a distance of 2-3Mb between probes, the apparent signal positioning reflects the true probe order on only 59-70% of chromatids. It was noted here that the proportion of cells with apparently

'correct' ordering of two probes varied not just with the probe distance (e.g. 100% for 937e12 and 925h10 at 5.5Mb distant as compared to 88.9% for probes 949b09 and 856h04 which are 1.65Mb apart), but also with particular probe sets (856h04 and 937e12 are also ~1.6Mb apart, and their correct signal order was only displayed in 35% of cells), although the variation might be a result of scoring only low numbers of cells.

YAC	CHROMOSOME 21 BAND LOCATION		
	MPI	THIS STUDY	ENSEMBL
949b09	q11.1-q21	q11.2	q11.2, 15.0Mb
858e10	?	q11.2	<i>proximal</i> q21.1, 15.8Mb
759d03	?	q11.2	<i>Ensembl localisation not possible as marker unknown</i>
856h04	?	q11.2-q21.1	q21.1, 16.6Mb
937e12	q11.1-q21	q11.2-q21.1	q21.1, 18.2Mb
925h10	q11.1-q21	q21.1-q21.2	<i>proximal</i> q21.2, 23.7Mb

Table 4-1 Comparison of 21q YAC FISH mapping results with Ensembl localisation of these sequences. Original MPI map data was obtained from the MPI website.

Mapping and Ordering of Chromosome 8-Specific Probes for Orientation of Duplicated Region and Breakpoint Definition in a Case of 8p Duplication

A literature search revealed no other reports of patients with the direct duplication of 8p reported in this study. Apparent duplications of 8p are most frequently inverted duplication deletions. These appear to be generated as a consequence of a maternal submicroscopic inversion delimited by the 8p olfactory receptor gene clusters in band 8p23. Inversion heterozygosity may cause susceptibility to unequal recombination, leading to the formation either of the inv dup del(8p) with deletion of the terminal region of 8p or of the reciprocal product, the +der(8p) (Giglio et al 2001, Shimokawa et al 2004). The only other report of a direct duplication proximal to 8p23 involves a familial dup(8)(p12p21.1) with mild MR as the only associated phenotypic effect (Moog et al 2000). As characterisation of this abnormality was by high resolution cytogenetic analysis and FISH with a microdissected probe for 8p12, the authors acknowledge that it was not possible to conclusively determine the exact breakpoints and nature of the duplication. In this study it has been possible, by exploiting the power of bioinformatics and availability of well-characterised BAC and PAC clones, to establish that the duplication of 8p reported here is direct, rather than inverted. The duplication includes centromeric sequences (at 43.1Mb from 8pter) but not an 8q sequence located ~0.6Mb from the centromere, and the distal breakpoint maps to 8p21.1~8p21.2, between 27.24Mb and 28.39Mb from 8pter. Although alpha satellite sequences were shown to be present interstitially on the short arm, there was no visible constriction. Accurate definition of breakpoints in all new cases of dup(8) will assist meaningful genotype/phenotype correlation in the future.

4.2 Detection of Submicroscopic Chromosomal Rearrangements

4.2.1 DG/VCFS Microdeletion Testing

Assessment of Detection Rates and Comparison with Other 22q11 Deletion Cohorts

A European collaborative study of 558 individuals with 22q11 deletion has been reported (Ryan et al 1997). A comparison of some of this data with the combined results of this work and subsequent diagnostic 22q11 deletion testing in this department is presented in Table 4-2. The figures exclude the TOF study.

The detection rate in this department, 11.7%, is broadly in line with published figures (13%, Berend et al 2000). Even in this patient cohort, many of whom were referred with unconfirmed DGS or VCFS clinical diagnoses, FISH testing for 22q11 deletion has been a highly informative test.

The proportion of deletions which could be confirmed as inherited appears lower in the present study. It is feasible that a suggestive family history, and hence inheritance, might have been a more frequent clinical feature among the first set of individuals to be tested, and hence over-represented, in the 1997 collaborative study. However this information is not provided. The data from the present work does confirm an excess of deleted mothers of affected children. The 1997 report suggested that this excess might in part reflect the fact that, where only one parent was tested, it was most commonly the mother, a finding which this work corroborates. The collaborative study also confirmed that the parental origin of deletion has no apparent effect on the phenotype.

High resolution G-banding is not routinely performed in this department, which explains the low proportion of cytogenetically visible deletions (12.9%). Detailed cytogenetics has demonstrated deletion in 30% of cases (Wilson et al 1992b).

Retrospective examination of the clinical data on 67 deleted patients of this department allowed assessment of presenting phenotypes and preparation of guidelines for prospective clinical diagnosis and FISH investigation (Tobias et al 1999).

	DGI	Ryan et al (1997)
Number of 22q11 deletions detected	100/855 (11.7%)	558
Inherited deletions	11/59 (18.6%)	81/285 (28%)
Maternal origin of inherited deletion	9/11 (81.8%)	61/79 (77%)
The proportion, where only one parent was studied, of mothers tested	14/19 (73.7%)	18/20 (90%)

Table 4-2 22q11 deletions detected in 855 index cases at the Duncan Guthrie Institute and comparisons with a collaborative study.

Phenotype/Genotype Correlation

When this study commenced it was recognised that DG/VCFS is not a straightforward contiguous gene syndrome. The severity and complexity of the phenotype does not correlate with deletion size (the three patients in this study who may have smaller deletions are not more mildly affected than others in the cohort) and it varies greatly even within one family (Scambler et al 1991, Kelly et al 1993). Discordant phenotypes have even been observed in monozygotic twins (Goodship et al 1995, Hatchwell 1996, Singh et al 2002).

All of the patient/parent combinations in this study showed intra-familial phenotype discrepancy. Phenotypic variability suggests the possibility of modifying genes at separate loci (Holder et al 1993) or, especially in the case of discordant twin phenotypes, a role for environmental factors or postzygotic events (Yamagishi et al 1999, Singh et al 2002).

Other Cytogenetic Abnormalities Found in Patients Referred for '22q Deletion Testing'

Of particular interest was the absence, in our patients referred for DG/VCFS deletion testing, of 10p deletions, the only other chromosome anomaly consistently reported in DG/VCFS. Only three 10p abnormalities (all in patients not referred for 22q11 deletion testing) were detected cytogenetically in this department during the course of this study: del(10)(p14), del(10)(p13) and t(9;10)(q22.1;p13).

Molecular studies suggest there is more than one DG/VCFS critical region on 10p (Dasouki et al 1997, Gottlieb et al 1998), though it is feasible that patients with variant deletions could also have a small deletion, rearrangement, or point mutation that maps in the shortest region of overlap, or that position effects might be involved. Because of the lack of definition of deletion intervals, widespread screening for microdeletions of 10p has not been adopted. One group developed a dual-probe FISH test for both 22q11 deletion and deletion at the 10p13p14 DGSII locus described in 1996 by Daw and colleagues (Berend et al 2000). Of 412 suspected DG/VCFS patients tested, 54 had 22q11 deletion but only one had deletion of 10p, and this was clearly cytogenetically visible. The

incidence of 10p13p14 deletion was therefore estimated as 1 in 200,000. Recently a cytogenetic and FISH study of 285 patients with suspected DG/VCFS identified 58 individuals with a 22q11 deletion and none with 10p deletion (Bartsch et al 2003).

Study of Patients with Isolated TOF

This study appeared to confirm the benefit of testing patients with non-syndromic TOF for 22q11 deletion. Four in 24 (16.6%) patients were deleted, compared to 2 in 40 (5%, Wilson et al 1992a) and 5 in 17 (29%, Goldmuntz et al 1993) in earlier studies.

Three deleted cases reported in the extended series of thirty three TOF patients in this study were thought to have no other DG/VCFS features (Trainer et al 1996). Other studies have concluded that subtle extracardiac anomalies are always present in association with apparently non-syndromic conotruncal lesions (Goldmuntz et al 1993). In one report, 0/107 patients with isolated TOF had 22q deletion. The only TOF cases in that study with deletion also had extracardiac anomalies, particularly dysmorphology (Amati et al 1995). Subtle dysmorphology, however, may not be useful for patient classification by clinicians other than clinical geneticists, and it is interesting that two of the VCFS patients with deletion in Amati's study were initially considered, prior to deletion detection, to have isolated TOF.

As a result of the TOF study, this department now routinely tests all prenatal samples with abnormal cardiac scan and all neonates with congenital heart defect for 22q11 deletion, even if, as in some cases, no other clinical features are recorded and 22q11 deletion testing is not specifically requested. Patients may exhibit other features of DG/VCFS that are not recorded on the request form. Also, although it has been shown that general 22q deletion screening of all paediatric patients with isolated cardiac abnormality is unlikely to disclose more deletions than screening based on presentation of a syndromic phenotype (Yong et al 1999), apparent isolated heart defect in an infant may later be re-diagnosed as DG/VCFS. Facial dysmorphology, LD and speech difficulties, for example, may only become obvious in the older child. Deletion has been demonstrated in five (9.1%) of the fifty five neonates tested.

Variant 22q11 Deletions

85-90% of patients with deletions have a common 3Mb deletion that is flanked by markers D22S427 in 22q11.21 and D22S801 in q11.23 (the 'typically deleted region', TDR, Carlson et al 1997, Edelmann et al 1999, Shaikh et al 2000), 8.5-12% have a variant proximal 1.5Mb deletion, and ~3% have a unique deletion or translocation (e.g. Levy et al 1995).

Both sc11.1 loci were shown to be included in one 1.5Mb deletion studied by detailed haplotype analysis (Edelmann et al 1999), and these authors claimed that all of the 3Mb and 1.5Mb deletions studied in a separate FISH investigation (Lindsay et al 1995) also involved both sc11.1 loci. However that FISH study of 54 VCFS patients actually did disclose one deletion which, in common with the possibly variant deletions reported in this work, apparently excluded one sc11.1 sequence.

By 1996 it was recognised that the phenotype could result from deletion distal to, and not overlapping, the DiGeorge critical region (Kurahashi et al 1996). There are at least two such non-overlapping deletion regions located distally in the typically deleted region. One includes HCF2, CHKAD26, and D22S935 but not ZNF74 (Kurahashi et al 1997). In another (McQuade et al 1999) the deletion is more proximal, including D0832 but not ZNF74 or HCF2. Other reported variants overlap these deletions (O'Donnell et al 1997, Garcia-Minaur et al 2002 etc.).

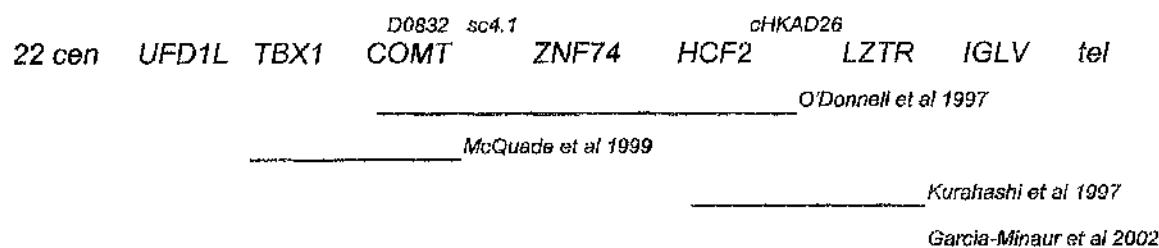


Fig.4-1 Positions of reported variant distal deletion regions in 22q11. Adapted from McQuade et al (1999) and Garcia-Minaur et al (2002). Not drawn to scale and some regions of possible (i.e. untested) deletion have been omitted for simplicity.

As well as the small deletions in the distal part of the 3Mb deletion, a deletion region associated with DG/VCFS has been reported that lies totally outwith and distal to the 3Mb TDR (Rauch et al 1999, Saitta et al 1999).

Homologous recombination involving four different blocks of low copy repeats (LCRs) are likely to be the cause of differently-sized and positioned deletions (Halford et al 1993b, Saitta et al 1999, Shaikh et al 2000). This mechanism for deletion/duplication formation has also been suggested for other recurrent chromosome rearrangements as found, for example, in Williams Syndrome (Dutly and Schinzel 1996) and in Charcot-Marie-Tooth disease Type 1A (CMT1A) and its reciprocal deletion, hereditary neuropathy with liability to pressure palsies (HNPP) (Stankiewicz et al 2003), as well as in non-recurrent rearrangements (Stankiewicz et al 2003). The breakpoint of the most common non-Robertsonian constitutional translocation, t(11;22), occurs in one of the 22q11 LCRs. Candidate genes for the del 22q11.2 phenotype in the shortest region of deletion (SRD) include clathrin heavy chain-like (*CLTCL*) and citrate transporter protein (*CTP*) (Gottlieb et

al 1997). A balanced translocation breakpoint in an individual with DG/VCFS has been reported in *CLTCL* (Holmes et al 1997). However the existence of other, phenotypically significant, non-overlapping regions, which now include a small (~20kb) deletion incorporating the *UFD1L* and *CDC45L* genes (Yamagishi et al 1999), means all the other transcription units in the TDR, of which there are about 30, still remain candidate causes. *TUPLE 1* was excluded from the SRD on the basis of a case with N25 deletion without *TUPLE 1* deletion, possibly the only reported instance of this type of variant deletion (Gong et al 1996). However, in the course of the diagnostic investigations reported in this study, patient KC was found to carry deletion of *TUPLE 1* but not N25. The only other reported incidence of *TUPLE 1* without N25 deletion may be a case described by Shaikh et al (2000). They found one of their 200 deleted patients had lost sequence 941 (c102g9) but not c19d3 (i.e. the proximal breakpoint was 50-100kb distal to N25). As N25 and *TUPLE 1* lie around 250kb apart (Gong et al 1996) presumably *TUPLE 1*, which was not tested, would be deleted in this case though N25 not deleted.

Currently Proposed Genetic Determinants of the DG/VCF Syndrome Phenotype

Evidence has grown that haploinsufficiency for *TUPLE 1* is at least in part responsible for the DG/VCFS phenotype. For example, *in situ* expression analysis shows chick and mouse *HIRA* homologues are expressed during embryogenesis in cells from which the main structures in DG/VCFS derive (Roberts et al 1997, Wilming et al 1997).

In addition it is now known that *HIRA* is homologous to the P60 subunit of human chromatin assembly factor 1 (Wilming et al 1997) and binds histones (particularly H2B and H4) and *HIRA*-interacting protein 3 (which binds H2B and H4), supporting its proposed role in regulation of local chromatin structure and hence gene expression (Lorain et al 1998). Its reported interaction with transcription factors *PAX3* and *PAX7* (Magnaghi et al 1998) endorses the theory that insufficient *HIRA* product could upset the assembly of multimolecular complexes involving homeodomain-containing protein binding factors required for normal embryonic development.

However, although extensive mapping, positional cloning, and sequencing of the 22q11 critical region (Dunham et al 1999) and mutation analyses of candidate genes in humans and deletion studies in mice have been performed, to date there is no evidence for a mechanism of haploinsufficiency that can fully explain the phenotype arising from 22q11 deletion. Only very recently has a gene in which mutation appears responsible for the 22q11 deletion syndrome been identified.

This is *TBX1*, one of the key candidate genes, which encodes a T-box transcription factor known to have an essential role in embryogenesis. Three different *TBX1* mutations (one

familial) have been found on analysis of ten CAF syndrome patients with no deletion at 22q11. These mutations are associated with a typical 22q11 deletion phenotype but no learning difficulties, a presentation consistent with the lack of *Tbx1* expression in the mouse brain, though a definitive clinical picture would require identification of large numbers of mutated patients (Yagi et al 2003, Baldini 2003).

Although it now appears that *TBX1* haploinsufficiency is responsible for a substantial subset of symptoms affecting 22q11 DG/VCFS patients, phenotypic variability was still present in patients with the *TBX1* mutations, suggesting that environmental factors and possibly interaction with downstream genes which are regulated by T-box transcription factors could be involved.

Investigators have also studied the association between variants and haplotypes of the remaining *TBX1* gene and the manifestation of congenital heart defects in 22q11.2 deletion patients, but common *TBX1* variants do not appear to be modifiers of congenital heart defect expression in these patients (Rauch et al 2004).

Two very recent studies, having employed nuclear scoring of FISH singlet or doublet (i.e. replicated) *TUPLE 1* probe signals, indicate possible roles in DG/VCFS aetiology for replication asynchrony. One publication reports comparable levels of asynchronous replication at 22q11.2 in all controls, translocation carriers and deleted individuals tested, and the authors propose that this asynchronous replication may favour LCR mispairing and generation of 22q11 deletion (Baumer et al 2004). The other study concentrated on the replication timing of the 22q11.2 region relative to a 22q13.3 control sequence and found that, in deleted DG/VCFS subjects, the remaining *TUPLE 1* locus in the non-deleted TDR replicates after the 22q13.3 locus, i.e. with a replication pattern opposite to that in normal controls. This suggests a possible alteration in the expression pattern of the genes in the non-deleted 22q11 DG/VCFS region (late replicating genes are inactive). *HIRA* insufficiency could be causing the altered organisation of 22q11.2 chromatin resulting in deregulation of replication timing, and the mechanism responsible for DG/VCFS could be a transcriptional repression of other genes, including *TBX1*, in the TDR (D'Antoni et al 2004).

Allele asynchrony at 22q13.3 (as reported by Baumer et al 2004) is incidentally noted, but no reference is made by D'Antoni and colleagues to any observed allele asynchrony at 22q11.2 in their undeleted control preparations.

22q11.2 Microduplication

Recent recognition of microduplication at 22q11.2 adds this to the growing list of complementary microdeletion/microduplication syndromes generated by reciprocal LCR-

mediated rearrangements (Edelmann et al 1999, Ensenaer et al 2003, Hassed et al 2004). Though generally detectable with non-routine high resolution G-banding, interphase FISH with *TUPLE 1* probe is recommended for definitive diagnosis. Reported duplications have the same centromeric breakpoint, at the proximal LCR implicated in the 3Mb DG/VCFS deletion, but variant distal breakpoints, generating segmental aneusomy of 3, 4 or 6 Mb. As with 22q11 microdeletion there is no correlation of duplication size with phenotypic severity and the phenotype, though it can be suggestive of VCFS, is very variable. However urogenital tract malformation and/or hearing loss is present in a significant proportion of patients (Ensenaer et al 2003).

A retrospective interphase FISH study of patients referred with possible DG/VCFS who had no 22q11 microdeletion is now being undertaken in this department. No microduplications have been found in the first forty patients analysed.

4.2.2 Wolf-Hirschhorn Syndrome Microdeletion Testing

WHS has been reported in association with *de novo* deletions and translocations and with familial translocations, of which the most frequently reported have been t(4;8) rearrangements (reviewed in Wieczorek et al 2000, Giglio et al 2002, Zollino et al 2004). The rate of familial translocations in WHS patients is now considered to be 15%, with *de novo* events occurring in ~85% of WHS patients (Wieczorek et al 2000).

At the start of this investigation, molecular genetic studies had defined a roughly 2Mb long WHS critical region, between loci D4S43 proximally and D4S90 (Johnson et al 1994). DNA studies performed in our proband and her parents by a collaborating laboratory (Department of Human Genetics, Western General Hospital, Edinburgh) allowed shortening of the critical region by exclusion of markers D4S111 and D4S115, and also found that this deletion was preceded by a paracentric inversion (Reid et al 1996). According to Ensembl the markers involved in this inversion lie distal to the most distal olfactory receptor (OR) gene cluster on 4p. Inversion polymorphisms in these OR clusters on 4p and others on 8p are known to predispose to the repeatedly reported t(4;8)(p16;p23) translocation which in an unbalanced form can result in WHS (Giglio et al 2002).

The minimal critical region has been narrowed to 165kb in band 4p16.3 (WHSCR1, Wright et al 1997) though a recent report of a patient with a deletion that excludes this region (Zollino et al 2003) has resulted in the proposal of a second critical region (WHSCR2) which comprises 300-600kb in 4p16.3 between D4S3327 and D4S98-D4S168, and is contiguous distally with the WHSCR1.

There has been some debate about whether deletion size correlates with phenotype severity and complexity, but patients with deletions of less than 3.5Mb have been reported to have a milder phenotype, usually with absence of major malformation (Zollino et al 2000, 2003). This 'minimal' WHS phenotype comprises typical facies, mental and growth retardation, hypotonia and seizures. Other authors who agree that there is some evidence for partial genotype-phenotype correlation in WHS conclude that birth weight, length, postnatal head circumference and the severity of MR correlate with the deletion size (Wieczorek et al 2000).

Though molecular studies can determine LOH, FISH can readily demonstrate translocation and carrier status and allows identification of all unbalanced forms of a cryptic translocation. The disclosure of the cryptic translocation described in this work is of continuing relevance to the family, and FISH provides a straightforward diagnostic tool. Recently, two phenotypically normal children of a known carrier (Fig.3-11 V-12, V-13) were tested with 4p and 11p telomere FISH probes (Cytocell) following confirmation that these probes were informative in their mother. This excluded carrier status in these individuals.

4.2.3 Microdeletion Testing in Rubinstein-Taybi Syndrome

During the course of this study the collation of data from a number of other investigations using the RT1 probe has allowed the frequency of microdeletion in RTS to be re-estimated at around 10%, rather than 25% (Petrij et al 2000). It is therefore understandable, especially as many of the referrals were not based on conclusive RTS diagnoses, that no microdeletions were detected in the small cohort of fifteen patients tested in this study. It has also been reported that microdeletions at the CBP gene can involve sequences other than the 3' RT1 locus. In 89 patients tested with five cosmids spanning the gene, eight microdeletions were found but four of these were 5' or interstitially located, and did not include RT1 (Petrij et al 2000).

As a result of the present study RTS testing with all five CBP-spanning cosmids is now available in this department.

Cytogenetically visible inversions and translocations have, more rarely, also been implicated in RTS, and truncating CBP mutations were found in 4 out of 37 RTS cases tested (Petrij et al 2000). Other types of mutation at CBP have also been linked to RTS (Kalkhoven et al 2003), however the cause of RTS remains unknown in ~80% of affected individuals, and a role for other genes is suspected.

4.2.4 Microdeletion Detection in Duchenne and Becker Muscular Dystrophy

FISH with exon-specific dystrophin cosmid probes provided an unequivocal, easy to interpret method of diagnosing DMD/BMD carrier status prior to, and during the introduction of quantitative PCR in this department, when no other conclusive test was available. The probes used were potentially informative for the majority of DMD/BMD microdeletions (Blonden et al 1991, Table 3-18). This approach gave a definitive result in 97 of 128 female relatives referred (75.8%), or in 94 of 111 female relatives referred (84.7%) if the 17 individuals with unconfirmed familial deletion are excluded. Among the many abnormalities detected was one at exon 52 missed by quantitative PCR analysis. This study also revealed some contamination of probe sequences relevant to this investigation and to the work of other authors (Bunyan et al 1995).

Quantitative PCR is now widely used for DMD/BMD deletion and carrier detection. In this department two multiplex assays detect 27 exons and the promoter region (using a modification of the method of Yau et al 1996). This technique has the advantage of being rapid and semi-automated, it tests more exons, therefore detecting over 70% of all mutations (>98% of all deletions) in the dystrophin gene, it does not require the familial deletion location without which FISH becomes very laborious, and it is capable of detecting duplications as well as deletions. Duplication detection by FISH, if at all achievable, would mostly only be possible using interphase FISH analysis. Quantitative PCR will however miss any chromosomal rearrangement, while FISH can detect interchromosomal and some intrachromosomal rearrangements, although none were found in this study. Denaturing high performance liquid chromatography (HPLC) and the Wave nucleic acid fragment analysis system (Transgenomic, Nebraska, USA) can indicate mutation-containing exons for subsequent targeted sequencing and has recently made mutation screening in DMD/BMD patients practicable in the diagnostic environment, raising the abnormality detection rate to over 92% (Bennett et al 2001).

Despite the availability of reliable and simple molecular means of DMD/BMD deletion and carrier detection, FISH remained the method of choice in some institutions (Calvano et al 1997, Voskova-Goldman et al 1997, Rosenberg et al 1998, Xiao et al 2003). A group from Texas, for example, has reported the use of 16 exon-specific cosmid probes to test 24 families (Ligon et al 2000). As FISH is still perceived as a valid and useful approach, a Multiprobe device with DMD/BMD exon probes has been developed (Cytocell) to allow simultaneous detection of 15 dystrophin loci in one experiment.

4.2.5 Screening for Cryptic Subtelomeric Rearrangement

Though the published prevalence of cryptic subtelomeric rearrangement in idiopathic MR varies from 0 to 23% in studies with varying sample sizes and selection criteria (Knight and Flint 2000), and an early report by the principal research group suggested an incidence of 7.4% (Knight et al 1999), a recent review of 20 studies incorporating 2500 subjects with MR suggests subtelomeric rearrangement may actually occur in approximately 5% of these patients (de Vries et al 2003). The phenotypically significant subtelomeric rearrangement detection rate was lower in the present study, at ~3.5%, but this is in accord with other reports, including a very recent study of subtelomeric screening by FISH in 94 individuals (Kirchhoff et al 2004). It may be that earlier studies with higher rates include a greater proportion of cases which are highly likely to be 'chromosomal' through the presence of a suggestive family history.

Even at the lower overall incidence of around 3%, subtelomeric abnormality may be the second most frequent recognised cause of moderate to severe MR after Down syndrome, supporting the value of this admittedly expensive test. The cost per informative test, when the testing of appropriate relatives of individuals with subtelomeric rearrangements is included, has been calculated as \$1600 (Knight et al 1999). However the value to the families concerned is immeasurable. The abnormal finding should have particular diagnostic impact in the large family of affected MC and SC. Their father, DM, has seven untested phenotypically normal siblings. MC and SC have four other phenotypically normal sibs and their affected nephew, DD, has two.

Results of FISH testing have provided evidence for the existence of novel microdeletion syndromes including del 1p36.3 (Shapira et al 1997, Heilstedt et al 2003) and del 22q13.3 (Precht et al 1998, Knight et al 1999). One patient with deletion at 1p36.3 and one with deletion at 22q13.3 were detected in the course of routine subtelomeric screening in this department. The patient with 1p36.3 deletion was dysmorphic and suffered from epilepsy, and the patient with 22q13.3 deletion had, in addition to behavioural problems, absent speech. This is consistent with the clinical features reported to be associated with these deletions. The patient with a deletion at 6q27 exhibited moderate to severe MR though it is recognised that this abnormality, especially unaccompanied by another segmental aneusomy, can be associated with a fairly mild phenotype even when considerable loss of genetic material occurs (de Vries et al 2003, Kraus et al 2003). Interestingly this patient had a family history of MR, although the deletion at 6q27 was *de novo*.

The authors of the original study invited reporting of further investigations to assist definition of appropriate clinical subcategories for testing (Knight and Flint 2000). Although the numbers studied here (14) are very small, subtelomeric rearrangement has not been found in any patient where the degree of MR or developmental delay has been described as 'mild', which is in accord with previous reports of low detection rates in such individuals (0.5%, Knight et al 1999). This is reflected in the notable increased focus on patients with moderate to severe MR in the diagnostic referrals to this department. A checklist of five criteria, including family history of MR, at least 2 facial dysmorphisms, and one or more non-facial dysmorphic feature or congenital abnormality, has been devised to assist patient selection for subtelomeric screening (de Vries et al 2001). All three of the subtelomeric rearrangements detected in the 100 cases reported here and four in the next 107 cases tested in this department were considered to be clinically significant on the grounds that (as suggested by Knight et al 1999) either the rearrangement is familial and the unbalanced derivatives segregate with MR (AS, MC), the rearrangements include regions previously found to be deleted in known MR syndromes (18q (MC), 1p36, 22q13.3, 6q27), or are so large that they are almost certain to have associated phenotypes (DR). Concurrent monosomy and trisomy is highly likely to be phenotypically significant. The remainder comprise abnormalities which are likely to be polymorphic, involving the 2q region frequently reported as such (e.g. in Knight and Flint 2000). Some of these were shown to be *de novo*, however, therefore a slim possibility of their clinical significance remains. Additionally, even where a segmental aneusomy is present in an unaffected parent (as could be shown for the deletions of 7p and 8p and one 2q deletion described here) the phenomenon of variable penetrance, as occurs with 22q11.2 deletions, could possibly be involved, or imprinting of the affected region. A similar 7ptel deletion case has recently been reported (Kriek et al 2004). Mosaicism in the unaffected parent with the segmental aneusomy, or a telomere position effect silencing sequences on the non-deleted homologue in the child, have been suggested as possible causes for diverse phenotypic effect in such cases (van Karnebeek et al 2002). More detailed investigation and definition of the regions involved in segmental aneusomies is required to assist interpretation of such FISH results. An 'in depth' comparison of the phenotypes associated with the abnormalities detected in this study with those associated with previously reported abnormalities involving these loci was not appropriate, as most of these reports involved much larger, cytogenetically visible aberrations. A recent review lists the phenotypes which have been associated with the reported cryptic and non-cryptic deletions of each subtelomeric chromosomal region (de Vries et al 2003) but, apart from the 1p, 22q and 6q deletions discussed above, all of the

phenotypically significant subtelomeric rearrangements detected in this department have involved aneuploidy at two loci, which is likely to complicate the phenotype.

Among the alternative approaches applied to subtelomeric screening have been high resolution cytogenetics (Joyce et al 2001) and short tandem repeat polymorphism (microsatellite) genetic markers (Colleaux et al 2001, Rio et al 2002).

Joyce and co-workers advocated that high resolution cytogenetics can detect most 'cryptic' subtelomeric rearrangements and FISH was only required to confirm positive findings. Such detailed cytogenetic analysis, however, can not be uniform in quality, and despite the findings of these authors a more sensitive, less subjective technique, is surely preferable. A subtle subtelomeric segmental aneuploidy (der(14)t(11;14)(p;q)) in a karyotypically normal child was recently disclosed by Multiprobe-T screening in this department, and only later determined to be cytogenetically visible when conventional cytogenetics was repeated (blind) by other cytogeneticists.

An advantage of genetic marker studies is that the sample, DNA, requires no cell culture, but the process can be cumbersome and may generate many more false positives than true positives, requiring customised follow-up for many of the screened cases (Biesecker et al 2002). Another disadvantage is that parental samples are required. However some aspects of the procedure can be automated and the microsatellite technique is capable of detecting uniparental disomy, although this phenomenon does not appear to be a significant contributor to the 'idiopathic MR' phenotype (reviewed in Biesecker et al 2002). Multicolour FISH has also been investigated, but found to lack sensitivity. In one study of 50 patients SKY detected only one of three subtelomeric rearrangements detected by FISH, and failed to show advantage in detecting any interstitial rearrangement (Clarkson et al 2002).

Other more readily automated, more sensitive subtelomeric screening approaches are currently in development as discussed below.

4.3 Comparative Genomic Hybridisation

The standard of CGH results in this study primarily depended on the quality of both the DNA and, as reported previously, the metaphase preparations. Commercial slides (Vysis) were of inconsistent quality, a finding confirmed by other investigators (Weiss et al 1999), but use of commercial control DNA was convenient and removed some variability from the technique.

The numbers of cases in the different categories of patients studied here are obviously too small to allow conclusions to be drawn from the abnormality detection rates observed.

However there were positive results, among these the detection of deletion in an individual with a balanced translocation involving other chromosomes. This phenomenon, overlooked abnormalities on cytogenetic analysis of cases with additional balanced abnormality, has been noted by other investigators (Kirchhoff et al 2001). Two of the six imbalances these authors detected in five cytogenetically balanced cases (Table 4-3) were at regions outside those involved in the translocation. Both of these aberrations, like the 1q25-q31 deletion in this study, were visible on subsequent examination of G-banded karyotypes, suggesting that the finding of a translocation may divert a cytogeneticist's attention from other abnormalities.

This CGH study also identified a 17p deletion and determined the region of duplication in an add(3) case, a task which would otherwise have required 'in house' culture of a range of chromosome 3 probes and sequential hybridisations.

One DNA sample (JG) repeatedly failed to produce CGH of analysable quality. Gel electrophoresis indicated that the proportion of high molecular weight DNA in this sample was low, but the OD₂₆₀/OD₂₈₀ ratio did not indicate protein contamination. The small volume of DNA available would, anyway, have precluded repeat phenol extraction and precipitation. More than the recommended amount of DNA will be used in labelling (as in the successful KD CGH experiments where the DNA was also degraded) if CGH with this sample is repeated, and alternative labelling methods will be attempted. Routinely assessing whether a DNA sample is degraded by preliminary electrophoresis makes an already lengthy CGH procedure less convenient in the diagnostic field. These studies suggest, however, that this should possibly be one of the first factors checked if the test, but not the reference, DNA fails to produce strong signal.

A case with a cytogenetic abnormality at 9p13 may have failed to exhibit imbalance on CGH because the chromosomal area of particular interest was adjacent to a pericentric region for which CGH may not be informative, providing interpretation problems. It has been noted (Kirchhoff et al 2001) that imbalances at 9p11 can, as in the case reported in this study, be detected in euploid DNA on rare occasions then 'disappear' on repeat of the analysis. These authors considered that they may represent technical artefacts. This study suggests that use of particular reference DNA/test DNA combinations may contribute to the effect. As expected the 21p deletion in case LF was also not detectable. Some CGH experiments, such as those with patient LS DNA, exhibited the locus-specific problems caused by unequal hybridisation of differentially-labelled test and reference DNAs to 1p32-pter, 16p, 19 and 22. These problems are now known to be associated with high GC content at these regions (Karhu et al 1997).

The possibility that imbalance is present in some of the cases studied, but outwith the resolution of CGH, can not be discounted. Empirical study using two different image

analysis systems and a threshold of 0.8 has determined CGH deletion resolution to be 10-20Mb, confirming earlier estimates (Bentz et al 1998), although cryptic subtelomeric translocations have been detected in three families with extensive histories of MR using this threshold, among them a subtelomeric deletion estimated at ~4Mb (Ghaffari et al 1998). However, rather than fluorescence ratio examination, the method involved comparison of fluorescence profiles and required very early "hands-on" CGH software, no longer commercially available, to allow user interaction at most stages of the analysis. This was even more time consuming than the use of current software.

Considering molecular cytogenetic alternatives to CGH, where the abnormality is cytogenetically visible but unresolvable, identification of extra chromosome material is also possible by microdissection of that region from metaphase spreads (Meltzer et al 1992) or by chromosome isolation by flow sorting (Blennow et al 1992) followed by FISH. Both techniques are limited in only analysing one chromosome region per hybridisation, and both require highly specialised instrumentation. M-FISH, the other global molecular cytogenetic technique, allows balanced rearrangement detection, but it requires reasonable quality patient chromosome preparations, it can miss small intrachromosomal aneusomies, and concerns have been raised regarding misinterpretation caused by overlapping fluorescence at the interface of translocated segments (Lee et al 2001). Multiprobe paint devices can offer simple alternative determination of imbalance resulting from interchromosomal rearrangement.

CGH has advantage over purely molecular methods, such as quantitative fluorescence (QF) PCR, in global detection of aneuploidies and unbalanced structural rearrangements, and is now widely applied to determination of quantitative genomic change in cases of subtle, suspected or apparent but unresolvable constitutional chromosomal aberrations (reviewed in Breen et al 1999) as well as in oncology studies. Refinements have included the use of alternative labelling techniques such as universal linkage system (ULS) chemical labelling (Alers et al 1999). This is particularly useful for the labelling of small fragment-sized DNA arising, for example, from archived tissue sections or generated by DOP-PCR, and may improve resolution, particularly at subtelomeric regions (Schoumans et al 2004).

Straightforward methods of CGH quality assessment have been suggested in a range of publications and were applied during this study. For example, mismatching the test and reference DNA sex and examination of the competitive hybridisation effect on the X chromosome to provide an internal control (Breen et al 1999). In the present study CGH was regularly capable of detecting dizygosity in individual cells at the X/Y pseudoautosomal region (~2.6Mb at Xp22.3 and Yp11.31, Rappold et al 1993) against a

background of effective Y imbalance in sex-mismatched test and reference DNAs. Also, if the limits of 95% or 99% confidence intervals of test versus reference CGH ratio profiles are used as thresholds, gains or losses can be identified when the 95% or 99% CI does not contain 1.0 (Weiss et al 1999).

Two modified CGH techniques have been introduced which improve CGH resolution by standardising interpretation. Both improve analytical quality by identifying inconsistently hybridised chromosomal regions. Four color CGH uses a second differentially labelled reference DNA as an internal standard (Karhu et al 1999). The alternative high resolution CGH (HR-CGH) approach employs software marketed by Applied Imaging. Instead of using fixed ratio thresholds and considering profiles deviating 15% or 20% from 1 (i.e. 0.85-1.15 or 0.8-1.2) to be aberrant, this involves comparison of profile 99.5% CI with a corresponding standard reference interval (an average of normal cases) which is wide at known variable areas (Fig.4-2). Where there is no overlap between the two intervals the chromosomal region is designated potentially aberrant (Kirchhoff et al 1998, 2000). As well as higher specificity, comparison of test case CIs with standard reference intervals also confers greater sensitivity (detection of 3Mb deletions) and therefore improved detection of mosaicism. No region needs to be excluded, and false positive results are infrequent. Among the patients tested in a major study using this approach were, as in this work, affected individuals with apparently balanced translocation and patients with apparent but unresolvable chromosomal abnormalities (Kirchhoff et al 2001, Table 4-3). In addition nineteen cases, seven of them cytogenetically cryptic, were studied to confirm a recognised abnormal karyotype. HR-CGH detected four of the seven cryptic aberrations, including two Prader-Willi/Angelman syndrome deletions. The undetected deletions were at DG/VCFS and WS regions. The other recognised abnormal karyotypes not clarified in the 2001 study were considered undetectable because they either contained no euchromatin, were normal variants, or mosaic.

A very recent publication by the same group reports an imbalance detection rate of 12% (51) in 424 karyotypically normal individuals with MR and dysmorphology (Kirchhoff et al 2004). Two-thirds of the abnormalities were interstitially and one third terminally located, and eight associated with recognised microdeletion syndromes including Smith-Magenis, blepharophimosis-ptosis-epicanthus inversus (BPES), 1pter, 2qter, and 22qter deletion syndromes.

Kirchhoff and co-workers acknowledge that not all of the abnormalities which could represent possible normal variants (through inheritance from a phenotypically normal parent) have been excluded from their HR-CGH detection figures. Adjustment of these figures to exclude six abnormalities in this category gives an HR-CGH detection rate of 10.6%.

A subset comprising 94 of these 424 patients were tested in a prospective study involving both HR-CGH and Multiprobe subtelomeric screening. Nine abnormalities were found, representing a detection rate of 9.6%. Eight of these abnormalities, two of which were terminal, were detected by HR-CGH (detection rate 8.5%). Three abnormalities, one of which was not detected by HR-CGH, were detected by subtelomeric screening (detection rate 3.2%). It is interesting that this group report a similar level of terminal deletions, 3-4% (Kirchhoff et al 2001, 2004), as found in the present study.

Kirchhoff and colleagues advocate use of HR-CGH in cases of dysmorphic MR and apparently balanced translocation, apparently balanced *de novo* translocation detected prenatally, clarification of complex structural rearrangement, and dysmorphic MR with normal karyotype (Kirchhoff et al 2001, 2004). They suggest HR-CGH for initial investigation of patients with idiopathic MR and dysmorphic features unless a family history implicates involvement of an unbalanced translocation, when subtelomeric screening should be used in the first instance. Subtelomeric screening should also be used for secondary investigation where HR-CGH is negative.

However these authors recognise that high resolution chromosome analysis could reduce the need for these time consuming and expensive procedures, and estimate that 30% of the abnormalities which they detected might have been found at the 500-750 band level even with no prior knowledge of their location.

HR-CGH software is now available in this department and will be used to analyse any repeat CGH tests for the cases in this study in which imbalance failed to be established.

INDICATION	NUMBER ANALYSED	ABNORMALITIES DETECTED
Affected, apparently balanced, <i>de novo</i> translocation	25	6 (21%)
Apparently balanced <i>de novo</i> translocation, PND	6	0
Clarification of abnormal karyotype	19	12
Confirmation of abnormal karyotype	19 (7 cryptic)	13

Table 4-3 Clinical cases analysed by HR-CGH (Kirchhoff et al 2001).

Further increase in the resolution of CGH has been provided by matrix CGH which uses cloned DNA arrays as a target instead of chromosomes and a laser scanner to record fluorescence intensities following hybridisation (Solinas-Toldo et al 1997, Fig.4-3). The construction and analysis of genomic microarrays has been reviewed (Carter et al 2002). Sensitivity is dependent on clone size and density, 3.5×10^3 clones giving 1Mb resolution. Commercial chips, however, are expensive, e.g. the Spectral Genomics array of 2500 clones costs £875 excluding labelling, though cost may reduce with increased demand. The alternative is to produce non-commercial diagnostic arrays. Researchers at the Sanger Centre, Cambridge have reported that an array of 500 clinically significant clones

(including oncogene, tumour suppressor, microdeletion and telomere sequences) plus band-specific sequences at 10Mb intervals could cost only £11- £44 per test, although a robotic 'arrayer' is required, at £75,000, for array production (Carter 2002).

This group also work with a 1Mb array of BAC clones which are logged in Ensembl and available from the BACPAC Resources Center, Children's Hospital Oakland Research Institute, Oakland, CA, or the Human BAC Resource at the Sanger Centre.

Use of this array for investigation of a chromosome derived from 21 in a child without Down syndrome physical features but with MR allowed characterisation of the over-represented region of 21. The tetrasomic region defined contains thirty four genes, among them sequences likely to contribute to MR in this condition (Rost et al 2004).

They have also screened 50 patients with MR and dysmorphic features on the 1Mb array and found twelve segmental aneusomies (Shaw-Smith et al 2004a). Five of these are inherited from phenotypically normal parents and so unlikely to be clinically significant, giving a detection rate of 14%. Though the numbers studied are small, this figure appears to correlate quite well with HR-CGH detection rates (10.6 -12%, Kirchhoff et al 2004), and suggests slightly improved sensitivity.

Similar work has been performed reproducibly validating the sensitivity of matrix CGH to detect 1.5-2.9Mb deletions (Vissers et al 2003). This group then tested a series of 20 patients with idiopathic MR and dysmorphism and demonstrated seven copy number alterations, five of which could be confirmed with FISH or high resolution arrays for the region of interest. Three of the five imbalances were considered likely to be genomic polymorphisms. The clinically significant abnormalities were 8.6 and 2Mb in size. To detect even more subtle abnormalities they are constructing microarrays completely covering the genome with an average resolution of 46kb (the BACPAC Resources Center's Human BAC Minimal Tiling Set).

The Cambridge group also use such higher resolution, overlapping, 'tilepath' clones on a secondary microarray to span any defined breakpoint. They have in addition developed a technique which, unlike standard CGH or microarray studies, allows the investigation of balanced rearrangements (Fiegler et al 2003). This array painting, as in reverse painting, involves flow sorting the derived chromosomes and their differential labelling but, instead of FISHing these to chromosomes, they are mixed and hybridised to a microarray of chromosome-specific clones. As the analyst surveys a set of these clones a reversal is apparent in the predominant fluor, defining the breakpoint. A clone with an intermediate ratio suggests it may span the breakpoint (Fig.4-4). Array painting is unlikely to be applied in diagnostic laboratories as it requires both demanding microarray and flow sorting technology and expertise.

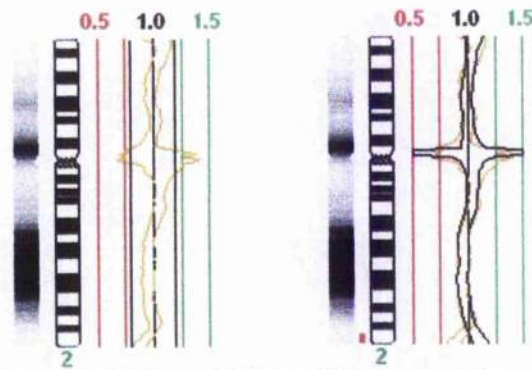


Fig.4-2 Comparison of CGH and HR-CGH interpretation.
CGH *left*: 99.5% confidence intervals (yellow) versus fixed ratio thresholds 0.8-1.2 (black).
HR-CGH *right*: 99.5% confidence intervals (yellow) versus the corresponding standard reference intervals (black) indicating loss at 2qter. Image courtesy of Applied Imaging.

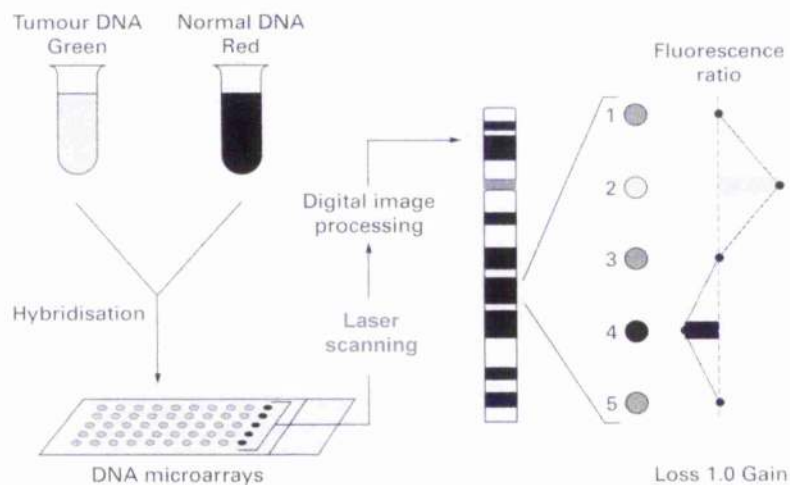


Fig.4-3 Schematic overview of the CGH microarray technique.
Tumour and reference DNA are labelled as for standard CGH and hybridised to fragments of DNA (instead of metaphase chromosomes) attached to a glass slide, ordered in arrays. Images of the fluorescent signals are captured and the green to red signal ratios are measured digitally for each target. From Weiss et al (1999).

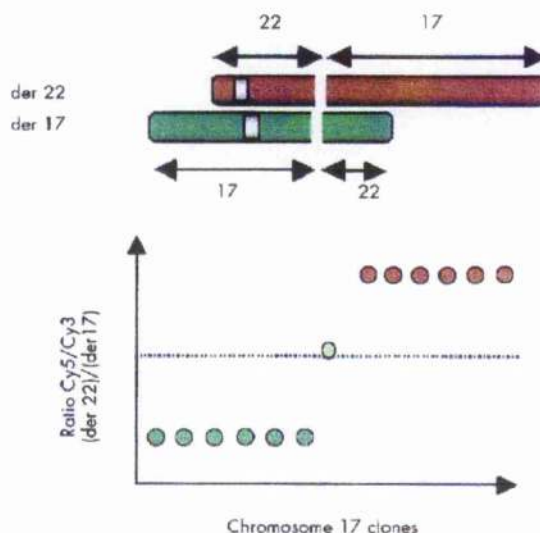


Fig.4-4 The basic principle of mapping translocation breakpoints by array painting.
Derivative chromosomes are flow-sorted, PCR-amplified, differentially labelled and co-hybridised to a chromosome-specific microarray. At the breakpoint site a reversal is apparent in the predominant fluor. From Fiegler et al (2003).

4.4 Primed *In Situ* Labelling

Assessment of PRINS with commercial kits confirmed the reliability of the Boehringer Mannheim system and that results could be obtained without a thermal cycler using this repetitive target kit. PRINS with 'in house' repetitive sequence primers was also successful, but no satisfactory results were obtained with primers for unique or very low copy sequences.

Two of the modifications assessed possibly offer potential for future PRINS optimisation. Pretreatment in Carnoy's fixative then 2xSSC appeared to increase penetrance and therefore signal. Also an alternative, relatively inexpensive, enzyme not previously reported in PRINS (Dynazyme) was shown, with low detergent, to give highly efficient amplification of repetitive primers D3Z1 and 267, requiring only one FITC detection layer to produce satisfactory non-cycling PRINS results.

The primary aim of these studies was to investigate the potential of PRINS for robust detection of unique target sequences. Statistical analysis of signal distribution had been necessary following chromogenic detection of *in situ* PCR with porcine unique sequences (Troyer et al 1994a,b), and the only report of unique sequence PRINS with fluorescence detection, Cinti et al (1993), had described Factor IX signal as 'barely detectable with conventional fluorescence microscopy' and stated that 'the signal to noise ratio and fading did not allow satisfactory photographic recording'. This suggested some amplification of signal would be needed, therefore for this study of Factor IX primers both PRINS and (as in Troyer et al 1994a,b), cycling PRINS were attempted. As no signal was produced in the absence of $MgCl_2$ with either Factor IX or control primers, placing the accuracy of the description of Cinti's method in question, PRINS was performed both using Cinti's approach and modifications, including use of $MgCl_2$. These tests also failed. Access to confocal laser scanning microscopy and signal enhancement for visualisation possibly improved the sensitivity of Cinti's system. Further modifications in the present study may have been beneficial, however solution PCR suggested specific amplification with these primers might not be consistently reproducible.

PRINS with DAZ and RBM1 primers was then investigated because these genes were believed to be present as low copy repeats and DAZ oligonucleotides had a large amplification product (1300 base pairs, Vogt et al 1996), which suggested that they could be useful as PRINS primers. Though solution PCR with Dynazyme worked well, PRINS was again unsuccessful. These sequences have actually had to be treated as 'unique' for the purposes of recent PRINS investigations (Kadandale et al 2002, see below).

Cycling invariably introduced damage and generally did not produce more signal than that achieved without cycling. In some experiments increased cycle numbers resulted in signal loss, suggesting diffusion might have been occurring. Published opinions on this effect differ. Though it has been reported that the products of PRINS cycles remain associated with their point of origin (Gosden and Hanratty 1993), Koch (1996) found that the majority of the extra DNA synthesised apparently did leave the site of synthesis producing increased background and less distinct signal. Komminoth (1994) also reported problems with diffusion during *in situ* PCR.

The initial restriction of PRINS to repetitive sequence detection meant it did not achieve widespread use. Also, PRINS rapidity offered little advantage as alphoid sequences can be detected (for PND or PGD) by FISH with only 2 hours incubation. One reported unique PRINS facility, differentiation of chromosome 13 and 21 alphoid sequences (Pellestor et al 1994, 1995b,c), might have significantly increased PRINS usage, particularly in PND. However preliminary PRINS investigations for this study found that signal with either 13 or 21 primers (though of differing strengths) was present both on chromosomes 13 and on chromosomes 21. It was subsequently confirmed that neither the 13 or 21 sequence variant is chromosome specific, but represents polymorphisms (Nilsson et al 1997, Yang et al 2001), which suggests that Pellestor's results could have depended on the individuals tested.

Among the findings in this study at variance with those of other reports is the optimal slide age for PRINS. Cinti et al (1993) stressed the importance of using 10 day-old slides for unique target PRINS, though use of such old slides would disadvantage diagnostic application. In this study no improvement was seen with unique primers on 10 day-old slides, instead, with repetitive primers, it was repeatedly found that 3 day-old slides produced optimal results and older slides reduced signal. Other authors (e.g. Gosden and Lawson 1994, Koch 1996) recommend the use of freshly prepared slides, certainly no older than 1 week. Also, PRINS results did not necessarily agree with published primer specificity, the pattern of signal appearance on 267-primed chromosomes regularly varying from that reported (Gosden and Hanratty 1993). For example, acrocentric chromosomes could display signal, even when signal was not present on both chromosomes 9 (as in 267 test 23).

It became clear from these experiments that optimisation of PRINS, even for the detection of repetitive target sequences, was not always straightforward - e.g. increasing annealing temperature to reduce signal might fail to do this and instead introduce speckled background. It is also more difficult to optimise PRINS than PCR as, generally, only a few experiments can be run simultaneously because of limited hotblock space.

These considerations, consensus that single-copy PRINS was, if achievable, never going to be a robust approach (Dr J. Gosden, MRC, Edinburgh, personal communication) therefore limiting its diagnostic applicability, and practical difficulties arising from changed work circumstances led to the conclusion of the PRINS component of this study in 1997. Repetitive sequence PRINS continued to be used by some investigators, as in the study of sequence organisation on chromosomes and extended chromatin (Therkelsen et al 1997, Shibasaki and Gosden 1997) and for sex determination in fetal cells in maternal blood (Orsetti et al 1998).

In 1999 and 2000 there were reports of PRINS adaptation and optimisation for the detection of single copy loci (Paskins et al 1999, Kadandale et al 2000). Paskins and colleagues used cycling PRINS on blood smears following ethanol fixation and microwave pretreatment. Due to the low frequency of cells with signal (40-50%) these authors did not, however, consider this approach to be applicable in a clinical diagnostic setting. During their investigations they discerned that the optimum size for primer pair products in cycling PRINS is 550bp, a longer product (900bp) dramatically reducing amplification efficiency. This may explain the poor cycling results in the present study as D3Z1 and all of the low/unique copy primers (except Factor IX) selected for use have larger amplification products.

More recently several presentations involving single-copy target PRINS were delivered at an international workshop on PRINS (Tharapel et al 2002, Kadandale et al 2002, Tharapel and Kadandale 2002, Cinti et al 2002). These included investigations of chromosomal regions studied in this thesis work, namely the DG/VCFS, RBM1, DAZ and dystrophin loci.

Three of these reports came from the same group at the University of Tennessee. Their modifications to the standard PRINS protocol included use of 1 day-old slides pretreated with 0.02N HCl, use of multiple primers for the same locus, single step annealing and protracted (30 minute) extension, use of TaqStart, a monoclonal antibody against Taq polymerase which prevents nonspecific amplification and formation of primer-dimers, and, as investigated in this work, TSA. Use of nested primers (for multiple DMD exons) had been considered for this study but not attempted due to lack of time.

Cinti et al (2002) also used no cycling, protracted extension and (unlike their 1993 report) 1 day-old slides, but not HCl treatment or TSA, incubating slides submerged in water to allow 'uniform diffusion of warmth'.

These 2002 single target PRINS reports suggested that PRINS might be about to have a fundamental impact on medical genetics. However a literature search covering the period since their publication, while revealing several reports including a new application of

PRINS in breakpoint refinement (Nirmakayalu et al 2003), demonstrates no obvious burgeoning of PRINS use. This may be because FISH has become established and routine in clinical cytogenetics laboratories and the range of commercial FISH probes has increased to cover the most frequently requested molecular cytogenetic investigations (Satinover and Schwartz 2002). Also unique target PRINS is probably more technologically demanding than FISH, the necessity for multiple primer sets and TSA makes the technique rather complex, and the ready availability of BAC and PAC probes provides a resource for FISH investigation of any region of the genome. PRINS may however continue to benefit research, e.g. in the investigation of the genomes of other species for which there are no, or only very limited, FISH probes.

5. CONCLUSION

Over the last decade molecular cytogenetic methods have greatly advanced cytogenetic resolution. We now stand on the brink of the full impact of even more powerful techniques aimed at the characterisation of segmental aneusomy, the most exciting of which is genomic microarray. This ultra high resolution CGH provides screening for gain as well as loss of genetic material, particularly relevant in the light of increased recognition of the clinical significance of microduplications which are not always detectable by subtelomeric screening and other FISH.

Genomic microarrays can be used for genome-wide screening or to investigate specific chromosomes (Buckley et al 2002) or chromosome sites (Yu et al 2003). Disease-specific probe sets to rival multiplex PCR may be developed. They have also been used to assess subtelomeric loci (Vissers et al 2003), however the significant number of cryptic interstitial abnormalities now being reported (Kriek et al 2004, Kirchhoff et al 2004) highlights the benefit of genome-wide, rather than targeted, screening of patients with idiopathic MR.

Microarray investigations will provide not only detection of microdeletion/duplication but also, because the arrays use characterised BACs therefore imbalances are directly linked to chromosome bands and genetic markers, instant detailed characterisation of the segmental aneusomy. This should greatly assist genotype/phenotype correlation and facilitate the detection of genes involved in physical and mental development.

Microarrays can provide a comprehensive screen, are high resolution, amenable to automation, rapid and sensitive, however a disadvantage is their current high cost which prevents their use on large sample numbers. Alternative, molecular, methods for targeted screening have different drawbacks, e.g. the presence of multiple primer pairs in a multiplex reaction reduces the robustness of PCR and the reliability of quantification, and the use of quantitative fluorescent PCR in a multiplex assay is limited by the spectral overlap of the fluorescent dyes used. New molecular techniques which increase the number of simultaneously analysable genetic loci include Multiplex Amplifiable Probe Hybridisation (MAPH, Armour et al 2000) and Multiplex Ligation-dependent Probe Amplification (MLPA, Schouten et al 2002). Both allow simultaneous amplification of a collection of target-specific probe sequences following hybridisation to patient DNA. MLPA is convenient in being a 'one-tube' assay, but probe development is more complex. These techniques currently detect copy number changes – deletions and duplications – at around 40 loci in a single reaction and some commercial kits are now available. In

addition to being less expensive, MAPH and MLPA have higher resolution (~100bp) than microarrays (BAC clones are 100-180kb) (Kriek et al 2004).

Amongst the first MAPH/MLPA probe kits to be developed were sets for subtelomeric regions (Sismani et al 2001, Hollox et al 2002, Rooms et al 2004) and DMD exons (White et al 2002). The DMD set provides comprehensive quantitative analysis of all 79 dystrophin exons, though appropriate exon-specific probes can be selected for carrier determination (White et al 2003). MLPA is now also available as an alternative to FISH for detection of segmental aneusomy in DG/VCF and Rubinstein-Taybi syndromes (Roelfsema et al 2005).

It has been suggested (Kriek et al 2004) that array CGH should be used for finding regions in the genome which harbour genes involved in particular diseases - microarrays have recently been exploited for this purpose by Vissers and colleagues (2004) - then, when these areas have been identified, less expensive MAPH/MLPA assays could be designed for their targeted diagnostic investigation. However it may be that, as with currently recognised recurrent segmental aneusomies, 'new' syndrome-associated imbalances will be associated with variable phenotypes. Global, rather than targeted, investigation will therefore continue to be preferable for many patients.

Additionally, the choice of targeted approach (microarray or MAPH/MLPA) is likely to be dependent on whether microarray prices reduce sufficiently, how rapidly the respective techniques are optimised for routine diagnostic use, and on the particular expertise in any department.

The Interpretation of Genetic Imbalances and the Role of Collaborative Databases

Segmental aneusomy of uncertain clinical significance is a frequent finding as determined, for example, in the FISH subtelomeric screening reported here and elsewhere and in microarray studies (Shaw-Smith et al 2004a). The advent of diagnostic microarray, MAPH, MLPA and HR-CGH has resulted in the institution of collaborative databases (ECARUCA, European Cytogeneticists Association Register of Unbalanced Chromosome Aberrations; CAC, Chromosome Anomaly Collection; DECIPHER, Database of Chromosomal Imbalance and Phenotype in Humans using Ensembl Resources) to help establish the clinical significance of any detected imbalance. DECIPHER will allow the user to determine whether a similar deletion or duplication has previously been reported by any other contributor to DECIPHER, compare a patient's phenotype with previous records, and visualise the known genes, putative genes and ESTs within the deleted/duplicated region. Pooling of data on molecularly-defined

segmental aneusomies will improve clinical definition of phenotypes and further assist genotype/phenotype correlation as well as definition of polymorphic variants.

This systematic analysis of polymorphisms should generate additional benefits, including insight into the flexibility of the human genome. It has been suggested that such large rearrangements, involving multiple genes, could serve as predisposing factors for multifactorial disorders (Vissers et al 2003).

Continuing Role of FISH in Determination of Segmental Aneusomy and Breakpoint Refinement

Choice of methodology is often determined by resources and expertise, as exemplified by the ongoing use of FISH, rather than quantitative molecular investigations, for DMD carrier testing in some departments (Ligon et al 2000).

Also the cost of digital imaging is now less than ten years ago, and developments such as automated interphase spot counting (Applied Imaging) and multicolor chromosome banding kits for individual chromosomes (Metasystems GmbH, Altlußheim, Germany) make FISH an increasingly attractive diagnostic and research tool, especially in combination with other molecular cytogenetic techniques.

Even with the introduction of new methodologies, a continuing role for FISH is assured, not just in, as currently, targeting well recognised syndromic sites but in mapping clones prior to their use in arrays (Shaffer and Bejjani 2004) and in validating the results of CGH and microarray (Lundsteen et al 2002, Vissers et al 2003) and MAPH/MLPA (e.g. Hollox et al 2002, Rooms et al 2004). Also, FISH screening, unlike microarray or MAPH/MLPA, is capable of detecting cryptic rearrangements in balanced carriers.

The potency of FISH has been increased by more straightforward access to large, accurately localised probe sequences and to online genome browsers (Ensembl, Birney et al 2004; UCSC genome browser, Kent et al 2002; various clone repositories including the Human BAC Resource), offering even the diagnostic laboratory the opportunity to precisely define breakpoints for more accurate genotype/phenotype correlation. Further definition of the cryptic chromosomal aberrations detected in this study was considered, but such comprehensive information and probe resources have only recently become accessible. Through investigation of the chromosome 8 and 21 rearrangements described here, experience in detailed mapping using these resources has been acquired and will continue to be utilised in the characterisation of chromosome abnormalities in the future.

The use of these new resources for the investigation of cytogenetically visible as well as cryptic aberrations is now being reported (Boylan et al 2004, Fantes et al 2004, Harewood

et al 2004, Willatt et al 2004, Collinson et al 2004, Novelli et al 2004, Johnson et al 2004a, Shaw-Smith et al 2004b), recent investigations in this department providing independent evidence for a CHARGE critical region at 8q (Johnson et al 2004b, manuscript in preparation).

It is apparent that the combination of molecular cytogenetic and microarray technology now available represents a highly effective means for revealing and accurately defining the many segmental aneusomies of clinical significance which undoubtedly await disclosure, particularly as aspects of these approaches may be amenable to automation and high-throughput screening. An associated benefit will be the uncovering of candidate genes for targeted mutation screening in appropriately affected individuals without segmental aneusomy, and the elucidation of genetic mechanisms involved in the aetiology of mental retardation and malformation syndromes. However, whether diagnostic services can take full advantage of these new technologies in the near future will, as ever, be heavily dependent on funding considerations.

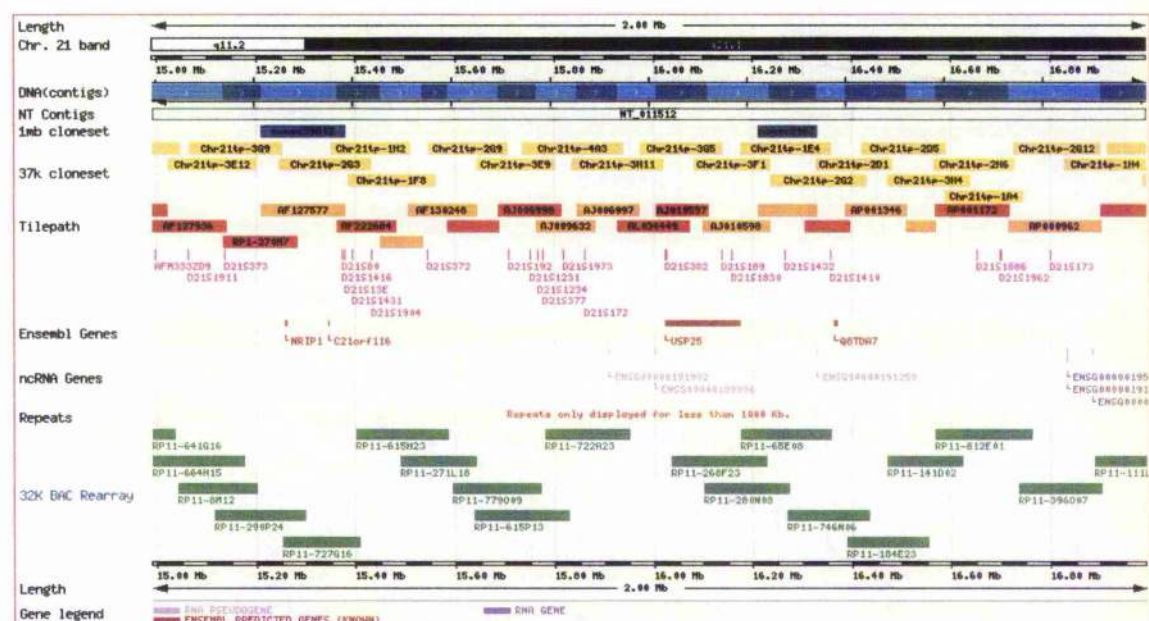


Fig. 4-6 The Ensembl Genome Browser showing 21q BAC clones (15-17Mb).

http://www.ensembl.org/Homo_sapiens

6. ELECTRONIC-DATABASE INFORMATION

BACPAC Resources Center, Children's Hospital Oakland Research Institute, Oakland, CA, <http://www.chori.org/bacpac/>

BACPAC Resources Center's human BAC Minimal Tiling Set, <http://bacpac.chori.org/pHumanMinSet.htm>

CAC (Chromosome Anomaly Collection), www.ngri.org.uk/Wessex/Register

Confidence interval calculation, <http://home.ubalt.edu.ntsbarsh/business-stat/otherapplets/ConfIntPro.htm>

DECIPHER (Database of Chromosomal Imbalance and Phenotype in Humans using Ensembl Resources), <http://www.sanger.ac.uk/PostGenomics/decipher/>

ECARUCA (European Cyto geneticists Association Register of Unbalanced Chromosome Aberrations), <http://www.ecaruca.net>

Ensembl Genome Browser, http://www.ensembl.org/Homo_sapiens

GenBank/NCBI Gene (links to NCBI nucleotide): <http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene>

Human BAC Resource, The Sanger Centre, Cambridge, <http://www.sanger.ac.uk/Teams/Team63/CloneRequest>

MAPH probe kit for subtelomeric regions, www.genetix.com/productpages/reagents/Genomics%20reagents/genecounter.htm

Max-Planck Institute for Molecular Genetics, <http://www.mpimg-berlin-dahlem.mpg.de>

MLPA kits for 22q11, subtelomeric regions and DMD exons, <http://www.mrc-holland.com>
MLPA kit for detection of microdeletions in RTS, <http://www.servicexs.com>

OMIM, <http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=OMIM>

Percentage of dystrophin deletions including any particular exon, http://www.dmd.nl/DMD_deldup_Leiden.html

Roswell Park Cancer Institute human BAC library, <http://bacpac.med.buffalo.edu>

STS markers for 32k rearray clones used in chromosome 21 breakpoint mapping, <http://genome.ucsc.edu/cgi-bin/hgTracks>

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8. APPENDICES

8.1 Appendix I - Details of Non-commercial Probe Sequences

8.1.1 Chromosomal Localisation of Unique Sequences

Endothelin 3-like Sequence

Endothelin is a vasoconstrictor released by the endothelium. There is evidence that mutation in this gene may be one of the causes of Waardenburg-Hirschsprung disease (Edery et al 1996). The PAC227 probe supplied for localisation had been isolated from the human genome mapping project (HGMP) PAC library, using a short endothelin sequence probe, by Ms K Warpeha and Dr A Hughes (The Department of Medical Genetics, Ophthalmology and Vision Sciences, Queen's University Belfast). PAC227 was expected to map to the same location (20q13.2-q13.3) as that determined, by restriction enzyme techniques in combination with somatic cell hybrids and also *in situ* techniques, for endothelin 3 (*EDN3* MIM 131242).

Myotonic dystrophy protein kinase-related Cdc42-binding kinase β

The p21 GTPases, Rho and Cdc42, regulate many cellular functions by binding to members of a serine/threonine protein kinase subfamily. These functions include the remodelling of the cytoskeleton in cell growth and differentiation. In rat, two of these p21 GTPase-regulated kinases, the myotonic dystrophy protein kinase-related Cdc42-binding kinases (α and β), phosphorylate nonmuscle myosin light chain, a prerequisite for the activation of actin-myosin contractility. The sequence supplied for mapping, named *CDC42BPB* or 82-h2, was a PAC clone of the human homologue of *MRCK β* isolated by our collaborators (Mr C Moncrieff and Dr K Johnson, Division of Molecular Genetics, Institute of Biomedical and Life Sciences, University of Glasgow) from the RP1 library (Roswell Park Cancer Institute, Ioannou and de Jong 1996).

Sequence data on *CDC42BPB* was deposited by our collaborators with the EMBL/GenBank Data Libraries under accession number AF128625.

Human Telomerase Reverse Transcriptase

Telomerase is a ribonucleoprotein complex which maintains chromosome length by adding DNA repeats to chromosome ends. In its absence, telomeres shorten with every cell division until, at a critical length, the cell becomes senescent. Loss of telomeric DNA

during cell proliferation may play a role in ageing and cancer (Counter et al 1992). Human telomerase is widely active during development but in adult tissues its activity is restricted to male germ cells, activated lymphocytes and stem cells of regenerating tissues. It is also detectable at high level in most immortal cell lines and human cancers. Telomerase is composed of an RNA template subunit, *hTR*, and a reverse transcriptase catalytic subunit *hTERT* which had, prior to this study, been tentatively mapped to distal chromosome 5p by PCR analysis of radiation hybrid panels (Meyerson et al 1997). Our collaborator, Dr WN Keith of the Department of Medical Oncology, University of Glasgow, initially provided a P1 clone containing the putative *hTERT* sequence for FISH mapping. As its localisation to 10q11.21~q11.22 (with a secondary site at 10q22.3) suggested that it could not be *hTERT*, another putative *hTERT* probe suitable for FISH mapping, 518C13, was obtained by our collaborators through screening the RP11 BAC library with the 3454bp cDNA insert of *hTERT* plasmid PGRN145 (Geron Corporation). Incorporation of the gene was confirmed by PCR amplification and sequencing of a 348bp promoter region.

Stearoyl coenzyme A desaturase 2

Stearoyl coenzyme A desaturase (*SCD*) is part of a membrane bound complex, catalysing the creation of *cis* double bonds in acyl-CoA derivatives of saturated fatty acids to produce mono-unsaturated fatty acids which are incorporated in cellular membranes. At the time of this mapping investigation rodent studies had identified two *scd* genes, *scd1* and *scd2*, with tissue-specific expression patterns. *Scd1* acts predominantly in liver and adipose tissue whereas *scd2* acts predominantly in the brain (Kaestner et al 1989). Human *SCD1* had been completely characterised by our collaborators, Ms K Dempsey and Dr J Craft of the Biological Sciences Department, Glasgow Caledonian University, and others (Zhang et al 1999) and the gene localised to chromosome 10. To attempt isolation of human *SCD2* our collaborators identified sequences which were *SCD*-like but not 100% homologous to *SCD1* from the GenBank database. One was amplified by PCR and identified as exon 4 of the putative *SCD2* gene. It was localised to chromosome 4 using PCR amplification of the sequence in a somatic cell hybrid panel. A LANL (Los Alamos National Library) chromosome 4 cosmid genomic library from the UK HGMP Resource Centre was then screened with the sequence to derive cosmid clones. Four clones (9, 17, 22 and 30) produced positive hybridisation to the *SCD2* sequence, and were supplied, at 0.18ug/ul, for regional localisation by FISH.

8.1.2 DG/VCF Syndrome Microdeletion Testing

All non-commercial probe sequences used were cosmid-incorporated and generously supplied by Professor P Scambler (Molecular Medicine, Institute of Child Health, University of London).

Early work involved cosmids sc4.1 (D22S134), which had been selected by Professor Scambler's group using molecular probe HP500, and sc11.1, which detected two loci, sc11.1a and b, at opposite ends of the commonly deleted 2-3Mb region (Carey et al 1992, Halford et al 1993b). The sc4.1 sequence was considered, at that time, to be included in all deletions. Cosmid sc11.1 showed high ISH efficiency and could provide indication of reduced deletion size, as the deletion in some individuals was believed to exclude the distal sc11.1b locus D22S139 (Lindsay et al 1995). On the recommendation of Professor Scambler the use of cosmid sequence E0472 (D22S933) was later adopted, because it was located relatively close to sc4.1 in the DG/VCF region and produced stronger signals.

Cosmid D0832 (D22S502), cloned from the proximal end of a YAC containing sc4.1, was primarily obtained for the investigation of a patient (EH) with particularly strong clinical indication of DG/VCF but no deletion of sc11.1, sc4.1 or E0472. It was the most proximal single-copy sequence available to us at the time, so was also used to confirm the absence of deletion in one individual from the series of TOF patients and to examine the extent of the deletion in two deleted patients from this group. No metaphase preparations remained from the other two deleted patients in this series to allow testing with D0832, and the probe was not more widely employed because of the introduction of H1012.

H1012 included candidate gene sequence *TUPLE 1* and was believed to be the most appropriate diagnostic indicator, therefore the use of other 22q11 cosmids was gradually discontinued.

Commercial probes which share sequences with H1012 were eventually used for testing. These were initially sourced from Cytocell (the coverslip-bound Chromoprobe-DG) and then Vysis (LSI *TUPLE 1* (22q11.2,3'*TUPLE 1*/D22S553/D22S609/D22S942) with LSI ARSA 22q13.3 control probe). N25 probe (LSI DiGeorge N25 (D22S75) Region Probe with LSI ARSA 22q13.3 control probe) was additionally employed for the most recent diagnostic testing.

8.1.3 Detection in a Large Pedigree of a Cryptic Chromosomal Rearrangement Involving the Wolf-Hirschhorn Syndrome Locus

Plasmid pK082, specific for chromosome region 4p16.3, was a 5.5kb EcoRI fragment in pBR328 and the gift of Dr JF Gusella (Harvard Medical School, Boston, Massachusetts). This sequence was a subclone of phage insert G8 and a polymorphic DNA marker linked to the Huntington's disease defect (Gusella et al 1983, MacDonald et al 1987).

The first 11p15.5 probe used was phins310, a 0.8kb sequence from the insulin locus cloned in the BamHI site of pBR327 (Bell et al 1981). This was replaced with phins311, an 11p15.5-specific, 8.6kb pBR322 clone of the insulin-like growth factor 2 sequence (Xiang et al 1987). Plasmids phins310 and phins311 are available from the American Type Culture Collection (ATCC 57400 and 59300 respectively).

8.1.4 Microdeletion Testing in Rubinstein-Taybi Syndrome

The RT1 probe is a 16p13.3-specific sequence (D16S237) cloned in cosmid pCpG. It was the generous gift of Drs Breuning and Petrij of the Department of Human Genetics, Leiden University.

RT1 covers only 29kb of the 3' end of the *CBP* gene, therefore later studies investigated the use of four additional cosmids to cover the entire 146kb *CBP* coding sequence.

Listing the most 3' (telomeric) sequence first, these were RT191, RT102, RT203 and RT166 (Petrij et al 2000). DNA and bacterial cultures were obtained from the Leiden Genome Technology Center (Human and Clinical Genetics, Leiden University Medical Center, The Netherlands, <http://www.LGTC.nl/>).

8.1.5 Duchenne and Becker Muscular Dystrophy Carrier Studies

Twenty nine bacterial stab cultures containing different DMD/BMD exon-specific cosmids, mostly from dystrophin deletion hotspots around exons 7 and 47, were obtained from Dr L Blonden and Dr JT den Dunnen (Department of Human Genetics, Leiden University) and Dr R Mountford and Nicola Robinson (North West Regional Genetics Service, St Mary's Hospital, Manchester). The Manchester cosmids had originally been sourced from the Imperial Cancer Research Fund Reference Library and screened with exon-specific primers for identification.

8.2 Appendix II - Details of Patient Samples

8.2.1 Chromosomal Localisation of Unique Sequences and Breakpoint Mapping

Mapping and Ordering of Chromosome 21-Specific Probes for Breakpoint Definition in a Case of Partial Trisomy 21

The case of partial trisomy 21 investigated in these studies was detected on chromosome analysis by the cytogenetic service at the Duncan Guthrie Institute following referral, by clinicians at the Southern General Hospital, Glasgow, of a heparinised blood sample from the affected neonate DP.

Patient LF, aged 28, was referred with mild MR, seizures and multiple physical abnormalities by clinicians of the West of Scotland Clinical Genetics Service. Cytogenetic analysis of a heparinised blood sample revealed a 46,XX,?del(21)(p) karyotype.

Mapping and Ordering of Chromosome 8-Specific Probes for Investigation of a Paracentric Duplication of 8p

Patient RM was referred by Dr K Ray of the Department of Community Health, Hamilton, for clinical genetic and cytogenetic examination in this department. In addition to mild global delay, difficulty sleeping, language and social problems, this three year-old child exhibited mild dysmorphic features including relative microcephaly, overturned ear helices, a notched incisor, slightly short palpebral fissures, proximally placed thumbs and overlapping toes. Toenails showed koilonychia and were dystrophic distally.

8.2.2 DG/VCF Syndrome Microdeletion Testing

Patients were referred for testing from hospitals served by the West of Scotland Clinical Genetics Service and from three other Scottish regions - Grampian, Lothian, and Dumfries and Galloway. Four of the investigations were performed on lymphoblastoid cell lines. The remaining samples were cytogenetic preparations from heparinised blood samples, two post-mortem tissue cultures, an amniotic fluid culture and a chorionic villus sample. Most tests were requested by a clinical geneticist or clinical geneticist in collaboration with a paediatrician or obstetrician. The tissue samples were referred by a paediatric pathologist. Karyotyping was, or had previously been, performed on all samples.

Three patients appeared to have variant deletions. Patient JF was referred with a patent ductus arteriosus, right aortic arch, mild learning difficulties, behavioural problems, and

facial dysmorphisms including hypertelorism and simple ears. Patient KA had interrupted aortic arch, VSD, absent thymus, hypocalcaemia, nystagmus, feeding problems, long slender fingers and abnormal facies, and patient HG presented with absent thymus, hypocalcaemia, dysmorphic features, velopharyngeal incompetence and failure to thrive.

8.2.3 Detection in a Large Pedigree of a Cryptic Chromosomal Rearrangement Involving the Wolf-Hirschhorn Syndrome Locus

The proband V-11 (Fig.3-11) presented in infancy with cleft palate, high nasal root, low set, posteriorly rotated ears, a well-demarcated philtrum, cupid's bow mouth, microcephaly, failure to thrive and poor feeding. A VSD, mild pulmonary valve stenosis, epilepsy, severe growth retardation (below the 10th centiles for height, weight and head circumference) and intellectual disability were later diagnosed.

The proband's second cousins once removed, a brother IV-8 and sister IV-10 aged 31 and 25 years respectively, had a similar pattern of abnormalities (severe cognitive disability, microcephaly, seizures, cleft palate and postnatal growth retardation). These sibs, like the proband, were originally thought to have an underlying diagnosis of Seckel syndrome.

Molecular cytogenetic investigations were performed on fixed metaphase preparations from lymphoblastoid cell lines (V-11, IV-11, IV-12) or heparinised blood samples. These samples were referred by Dr J Tolmie of this department, Dr E Thomson of the Kennedy-Galton Centre, Northwick Park Hospital, Harrow (III-6, IV-7, IV-16, IV-17) and Dr D Fielding of the Countess of Chester Hospital, Chester (IV-8 and IV-10).

8.2.4 Microdeletion Testing in Rubinstein-Taybi Syndrome

Fifteen patients considered to have a possible clinical diagnosis of RTS on the basis of characteristic physical and intellectual disabilities were referred by clinical geneticists of the West of Scotland Clinical Genetics Service. All had a normal karyotype. Three were also tested, and shown to be undetected, with DG/VCFS probe. Two of these three and two others subsequently underwent subtelomere screening with negative results.

8.2.5 Duchenne and Becker Muscular Dystrophy Carrier Studies

Patient heparinised blood samples, from which cytogenetic preparations were made, were referred for FISH testing with DMD/BMD cosmid by clinical geneticists of the West of Scotland Clinical Genetics Service and other Scottish Clinical Genetics Services in Grampian, Tayside and the South East.

Multiplex PCR was used by the molecular genetics division of this department to screen DNA samples from the male patients for deletion of DMD/BMD exons. Creatine kinase levels were determined for the female patients by the biochemical genetics division.

8.2.6 Testing with Cytocell Multiprobe-T for the Detection of Cryptic Subtelomeric Rearrangements in Idiopathic MR

All samples were received from Scottish Clinical Genetics Services except the blood samples from members of the family with cryptic t(10;18). These were referred by David MacGregor and Dr S Moore of the Janeway Genetics Clinic, St John's, Newfoundland, Canada.

The physical abnormalities present in 99 of the 100 patients tested included, among others, minor facial and distal dysmorphism (e.g. brachy-, clino- or camptodactyly, simian crease), reduced growth (most commonly, measurements of -2 SD) and dysmorphisms affecting external genitalia such as undescended testis, inguinal hernia and micropenis. The spectrum of abnormalities observed at two years in patient DD of the t(10;18) family included moderate to severe developmental delay, failure to thrive, small head circumference (5th percentile), VSD, and like his uncles, short stature (5th percentile), hypotonia and joint laxity, asthma, reduced pigment, gastro-oesophageal reflux and recurrent aspiration (resulting in pneumonia). Dysmorphic features noted were malar hypoplasia, epicanthus (like SC), flat nasal bridge with short nose and anteverted nares, well-developed philtrum, wide down-turned mouth, low-positioned and spatulate thumbs, prominent fingertip pads on some digits and blue eyes with brushfield spots (as in SC). Previous FISH testing for DG/VCF and Smith-Magenis syndromes had proved normal.